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<b>(54) Title:</b> NON-HEMATOPOIETIC CELLS, INCLUDING CARDIOMYOCYTES AND SKELETAL MUSCLE CELLS, DERIVED FROM HEMATOPOIETIC STEM CELLS AND METHODS OF MAKING AND USING THEM  <b>(57) Abstract</b> <p>The present invention provides a process of promoting differentiation of a stem cell into a cardiomyocyte or skeletal muscle cell, comprising the steps of obtaining a stem cell, which is preferably a hematopoietic stem cell, with cardiomyocyte or skeletal muscle cell potential from a donor and contacting the stem cell with a growth factor or combination of growth factors. The invention also provides a population of cardiomyocytes or skeletal muscle cells derived using the process and the nonembryonic stem cells having cardiomyocyte or skeletal muscle cell potential or embryonic or nonembryonic hematopoietic stem cells. Further provided is a composition, comprising the stem cells and a combination of growth factors in amounts and conditions to promote the differentiation of the stem cells into cardiomyocytes or skeletal muscle cells. Also provided are methods of using the cells of the present invention.</p>		

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NON-HEMATOPOIETIC CELLS, INCLUDING CARDIOMYOCYTES AND  
SKELETAL MUSCLE CELLS, DERIVED FROM HEMATOPOIETIC STEM  
CELLS AND METHODS OF MAKING AND USING THEM

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This invention was made with government support under RO1 HL55923  
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The government has certain rights in the invention.

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BACKGROUND OF THE INVENTION

**Field of the Invention**

The present invention is related to specific cell types, including cardiomyocytes  
and skeletal muscle cells, derived from either nonembryonic stem cells, such as  
15 hematopoietic stem cells, or embryonic hematopoietic stem cells, the method of  
derivation, and therapeutic uses of the stem cells and the cells derived from the  
nonembryonic stem cells and the embryonic hematopoietic stem cells.

**Background Art**

20 Certain cells, like skeletal muscle cells and cardiomyocytes, lack the ability to  
proliferate *in vivo* or *in vitro*. Accordingly, uses of these non-proliferating cells have  
been limited by the lack of a convenient, renewable source.

For example, cardiac disease is the number one cause of mortality and  
25 morbidity among the United States population. Because the adult heart does not  
exhibit a regenerative capacity, this dysfunction is mainly due to the loss of  
cardiomyocytes and the infiltration of scar tissue (Soonpaa *et al.*, 1995). Although  
some success has been reported in promoting adult cardiomyocyte proliferation (U.S.  
Patent No. 5,543,318), adult cardiomyocytes *in vitro* do not possess the capacity to  
30 proliferate. Thus, the loss of cardiomyocytes is irreversible.

The only treatment currently available for replacing diseased myocardial tissue is organ transplantation. Because of the limited availability of donor hearts, however, relatively few potential recipients can benefit from heart transplantation. Even if the problems with cardiac availability were overcome, the high costs involved in this procedure and the radical nature of the surgery would still limit organ transplantation to only those patients with end-stage diseased hearts. Thus, alternatives to organ transplantation are needed.

Grafting of healthy cardiomyocytes into a recipient's heart has been proposed as an alternative to organ transplantation. The feasibility of grafting cells into cardiac tissue has been shown in animal studies using a variety of cell populations, including skeletal myoblasts, cardiac tumor cell lines, fetal cardiomyocytes, and cardiomyocytes derived from embryonic stem (ES) cells (Klug *et al.*, 1996; Koh *et al.*, 1993a; Koh *et al.*, 1993b; Koh *et al.*, 1995; Soonpaa *et al.*, 1994). In addition, transfected embryonic stem cells have been differentiated into cardiomyocyte lineage and have successfully been incorporated into cardiac tissue (U.S. Patent No. 5,733,727). Success using these approaches in various animal models has confirmed the potential of intracardiac grafting as a therapeutic intervention in diseased hearts (Delcarpio and Claycomb, 1995; Li *et al.*, 1996a; Li *et al.*, 1996b; van Meter Jr *et al.*, 1995). A finding common to these studies is that, regardless of the animal model used, the success of these experiments is dependent upon the developmental stage of the injected myocytes. Only fetal or neonatal cells demonstrated stable integration into adult hearts. In contrast, adult cardiomyocytes will not successfully incorporate into cardiac tissue (Reinecke *et al.*, 1997). It is postulated that the age dependence of the grafted cells is due to the proliferative capacity of young cardiomyocytes (Klug *et al.*, 1996).

Embryonic cardiomyocyte grafts as used in treating heart disease, however, present certain problems. Because of the ethical issues involved, as well as various technical and availability limitations, the use of embryonic tissue is not practical. Nor

can adult cardiomyocytes practically be harvested, even if proliferation can be promoted *in vitro*. Furthermore, these cardiomyocyte grafts from embryonic or nonembryonic donors still necessitate the use of chronic immunosuppression to prevent rejection of the graft by the recipient. Thus, alternative sources of cardiomyocytes  
5 capable of stable integration into the adult heart are needed. These sources ideally would provide the potential of deriving the donor cells from the recipient to avoid graft rejection.

Similarly, regeneration of adult skeletal muscle is also very limited because  
10 mature multinucleated myocytes show little proliferative capacity. Embryonic myoblasts and satellite cells have generally been used as a source of skeletal muscle cells. These sources, however, present the same limitations as the available sources for cardiomyocytes.

## 15 SUMMARY OF THE INVENTION

The present invention provides a method of promoting differentiation of a hematopoietic stem cell into a cell of non-hematopoietic lineage, comprising the steps of obtaining a hematopoietic stem cell with non-hematopoietic potential from an  
20 embryonic or nonembryonic donor and contacting the stem cell with a growth factor or combination of growth factors, thereby promoting differentiation of the hematopoietic stem cell into a cell of non-hematopoietic lineage. Preferably, the hematopoietic stem cell is a bone marrow cell or a blood cell.

25 The present invention further provides a process of promoting differentiation of a stem cell into a cardiomyocyte or skeletal muscle cell, comprising the steps of obtaining a stem cell with cardiomyocyte or skeletal muscle cell potential from a nonembryonic donor or a hematopoietic stem cell from an embryonic donor and contacting the stem cell with a growth factor or combination of growth factors, thereby

promoting differentiation of the stem cell into the cardiomyocyte. Preferably, the stem cell is a bone marrow cell or blood cell, more preferably a hematopoietic stem cell, and even more preferably a myeloid-lymphoid stem cell (including, for example, a myeloid-lymphoid stem cell intermediate). Further provided is a composition,

5 comprising stem cells with cardiomyocyte or skeletal muscle cell potential derived from a nonembryonic donor or hematopoietic stem cells with cardiomyocyte or skeletal muscle cell potential derived from an embryonic donor and *Wnt* (e.g., a member of the class *Wnt5a*, including e.g., *Wnt11*), retinoic acid, basic fibroblast factor, fibroblast growth factor-4, dexamethasone, bone morphogenic factor -4, and either acidic

10 fibroblast growth factor or endothelial cell growth factor in amounts to promote the differentiation of the stem cells into cardiomyocytes. Also provided is the composition further comprising stem cell factor, endothelin-1, transforming growth factor beta, interleukin (including, e.g., IL-2, IL-10 and IL-15), and a PKC inhibitor (e.g., sphingosine). The invention also provides as an alternative embodiment, a

15 composition, comprising stem cells with cardiomyocyte or skeletal muscle cell potential derived from a nonembryonic donor or hematopoietic stem cells from an embryonic donor and stem cell factor, transforming growth factor beta, endothelin-1, and either basic fibroblast growth factor or endothelial cell growth factor in amounts to promote the differentiation of the stem cells into skeletal muscle cells. Also provided

20 is the composition further comprising retinoic acid, interleukin (including, e.g., IL-2 and IL-15), insulin-like growth factor-1, platelet derived growth factor dexamethasone, angiotensin II, 3,3',5-triiodo-L-thyronine, and hyaluronic acid.

The present invention also provides a method of determining whether

25 nonembryonic stem cells or embryonic hematopoietic stem cells of unknown cardiomyocyte or skeletal muscle cell potential can be promoted to differentiate into cardiomyocytes or skeletal muscle cells, comprising the steps of contacting the stem cells with a growth factor or combination of growth factors under conditions that allow

the cells to differentiate, and determining the presence of differentiated cells having one or more characteristics of cardiomyocytes or skeletal muscle cells.

The invention also provides a method of treating a subject with heart injury or disease, comprising transplanting the cardiomyocytes of the present invention or the stem cells with cardiomyocyte potential into the cardiac muscle of the subject, thereby improving cardiac function. Also provided is a method of treating a subject with muscle injury or disease, comprising transplanting the skeletal muscle cells of the present invention or the stem cells with skeletal muscle cell potential into the skeletal muscle of the subject, thereby improving skeletal muscle function. Thus, an object of the present invention is to provide a source of cardiomyocytes or skeletal muscle cells for transplantation using bone marrow or blood cells in order to overcome problems with the prior art. The issues involved in promoting differentiation from bone marrow or blood cells are not trivial, as the subpopulation of cells that possess cardiac or skeletal muscle cell potential are relatively few in number and their cellular environment within the blood or bone marrow is not normally conducive for cardiac or skeletal muscle cell differentiation.

#### DETAILED DESCRIPTION OF THE INVENTION

20

The present invention provides a method of promoting differentiation of a hematopoietic stem cell into a cell of non-hematopoietic lineage, comprising the steps of obtaining a hematopoietic stem cell with non-hematopoietic potential from a nonembryonic or embryonic donor and contacting the stem cell with a growth factor or combination of growth factors, thereby promoting differentiation of the hematopoietic stem cell into a cell of non-hematopoietic lineage. Preferably, the hematopoietic stem cell is a bone marrow or blood cell, including myeloid-lymphoid stem cells, myeloid-lymphoid stem cell intermediates, and monoDC progenitor (i.e., the monoblast or the DC progenitor). More preferably, the hematopoietic stem cell is a myeloid-lymphoid

stem cell. As used throughout, "hematopoietic stem cells" include multipotent hematopoietic stem cells, BFU-Es, GM-CFUs, GEMM-CFCs, CFCs, B1-CFCs, NK progenitors, dendritic progenitors, T progenitors, or B progenitors. As used throughout, a "cell of non-hematopoietic lineage" includes non-blood and non-lymph  
5 cells and precursors thereof, including, but not limited to, myoblasts and myocytes (both cardiomyocytes and skeletal muscle cells). It is understood that the stem cells of the present invention can also be directed to differentiate into other cells of non-hematopoietic lineage using similar procedures as well as into cells of hematopoietic lineage.

10

As used throughout, the step of contacting the stem cell with a growth factor or combination of growth factors can be performed using a defined culture medium or alternatively can be performed either *in vivo* or *in vitro* by contacting the stem cell with either cardiac tissue, skeletal muscle tissue, or any other tissue that produces  
15 environmental cues to promote differentiation of a hematopoietic cell or other stem cell into a cell of non-hematopoietic cell lineage.

Thus, the present invention provides a process of promoting differentiation of a stem cell into a cardiomyocyte or skeletal muscle cell, comprising the steps of  
20 obtaining a stem cell with cardiomyocyte or skeletal muscle cell potential from a nonembryonic donor and contacting the stem cell with a growth factor or combination of growth factors, thereby promoting differentiation of the stem cell into the cardiomyocyte or skeletal muscle cell. "Stem cell with cardiomyocyte or skeletal muscle cell potential" does not include embryonic stem cells, mesenchymal stem cells,  
25 satellite cells, or QCE-6 cells.

By "cardiomyocyte" is meant any cell in the cardiac myocyte lineage that shows at least one phenotypic characteristic of a cardiac muscle cell. Such phenotypic characteristics can include expression of cardiac proteins, such as cardiac sarcomeric or



myofibrillar proteins or atrial natriuretic factor, or electrophysiological characteristics. Cardiac sarcomeric or myofibrillar proteins include, for example, atrial myosin heavy chain, cardiac-specific ventricular myosin heavy chain, desmin, N-cadherin, sarcomeric actin, cardiac troponin I, myosin heavy chain, and Na-K-ATPase. Electrophysiological characteristic of a cardiomyocyte include, for example, transient  $K^+$  channel currents, and acetylcholine and cholera toxin responses. Similarly, by "skeletal muscle cell" is meant any cell in the skeletal muscle cell lineage that shows at least one phenotypic characteristic of a skeletal muscle cell. Such phenotypic characteristics can include expression of skeletal muscle proteins, such as skeletal muscle-specific transcription factor MyoD or skeletal muscle-specific myosin. In addition, skeletal muscle electrophysiological characteristics and morphologic characteristics are included. For example, fusion into a multinucleated striated fiber is a phenotypic characteristic of skeletal muscle.

By "promoting differentiation of the stem cell into the cardiomyocyte or skeletal muscle cell" is meant inducing a stem cell to follow a cardiac muscle or skeletal muscle lineage such that the stem cell gives rise to progeny that exhibit at least one phenotypic characteristic of a cardiomyocyte or skeletal muscle cell.

By "nonembryonic" is meant fetal or postnatal. The embryonic period is considered to be early prenatal development, and specifically, in the human, the first eight weeks following fertilization. One skilled in the art would recognize that the equivalent period in other mammalian species would constitute the embryonic period. The present invention excludes embryonic stem cells, embryonic cardiomyoblasts or cardiomyocytes, and embryonic skeletal myoblasts and myocytes. Certain embodiments of the present invention, however, utilize embryonic hematopoietic stem cells.

The cardiomyocyte derived by the present method is noncontracting but contains contractile elements. By "noncontracting" is meant non-beating. More specifically, "non-beating" or "noncontracting" means the absence of spontaneous contraction. The cardiomyocytes, however, are capable of contraction upon  
5 inducement. Methods of inducing contracting include introduction of the derived cardiomyocyte into spontaneously beating cardiac muscle *in vivo*, whereupon the cardiomyocyte is capable of electronic coupling with the spontaneously contracting cardiac muscle and integrating into the syncytium of the cardiac muscle, thereby becoming a contracting cardiac muscle cell. Alternatively, contraction can be induced  
10 *in vitro* by co-culturing the derived cardiomyocytes with contracting cardiomyocytes isolated from embryonic, fetal, or postnatal heart. Contraction can also be induced by changes in ion concentration (e.g., by elevated  $K^+$ ), by mechanical stimulation, or by electrical stimulation (e.g., Ingber et al., 1991; Johnson et al., 1993; McDermott et al., 1985; and Simpson et al., 1994). One skilled in the art would recognize that cardiac  
15 sarcomeric and myofibrillar proteins are not necessarily organized into complete myofibrils in cardiomyocytes. For example, it is well known in the art that well-developed cardiomyocytes lose their organized myofibrils when maintained in culture for an extended period of time. The myofibrillar organization is re-established, however, upon contacting the cardiomyocytes with contracting cells (e.g.,  
20 cardiomyocytes) either *in vivo* or *in vitro*. The same is true of the noncontracting cardiomyocytes of the present invention, i.e., they are capable of complete myofibrillar organization and contraction when contacted with contracting myocytes. Other cell types that can induce myofibrillar organization and contraction include, for example, blastodermal cells or neuronal cells (e.g., neuroblasts).

25

By "stem cell" is meant a non-immortalized cell that possesses the capability of dividing and producing progeny that include mature, differentiated cells. The stem cells used in the present method have non-hematopoietic potential, including for example, cardiomyocyte potential or skeletal muscle potential. By "cardiomyocyte

potential" or "skeletal muscle cell potential" is meant the ability to give rise to progeny that can differentiate into a cardiomyocyte or skeletal muscle cell under specific conditions. Examples of stem cells with cardiomyocyte or skeletal muscle cell potential include certain bone marrow or blood cells and certain hematopoietic stem cells, including myeloid-lymphoid stem cells, myeloid-lymphoid stem cell intermediates, monoDC progenitor (i.e., the monoblast or the DC progenitor). Thus, in the present method, the stem cell used to derive the cardiomyocyte or skeletal muscle cell is preferably a bone marrow or blood cell, and, more preferably, a hematopoietic stem cell, and even more preferably a myeloid-lymphoid stem cell. Preferably, the hematopoietic stem cell from which the present cardiomyocyte or skeletal muscle cell is derived is a nonadherent cell. By "non-adherent" is meant a cell that does not adhere to tissue culture plastic or that adheres loosely, so as to be easily dislodged by gentle pipetting.

15 Hematopoietic stem cells as used in the present invention can be isolated from nonembryonic donors from, e.g., the bone marrow, the spleen, the liver, and peripheral blood. Hematopoietic stem cells can be isolated from an embryonic donor from the aorta-gonad-mesonephros area, the spleen, the liver, and the bone marrow.

20 One skilled in the art would know that the bone marrow, for example, comprises a heterogeneous population of cells at different stages of maturity. Separation of more mature cells from stem cells can be performed using any one of the techniques well known in the art, including cell density gradients (e.g., Ficoll-Paque), cell sorting using cell surface antigens (e.g., with a fluorescence activated cell sorter),  
25 or by magnetic beads (StemSep Technologies) which bind cells with certain cell surface antigens. These methods can be combined to yield a relatively pure population of hematopoietic stem cells for use in the present invention. For example, the Ficoll Paque method and the magnetic bead method can be combined to yield hematopoietic stem cells with a purity of greater than about 85% and optimally of about 99% purity.

Hematopoietic stem cells can also be isolated from peripheral blood using similar techniques. See Care *et al.*, 1999); Janowski-Wieczorek *et al.*, 1999.

The stem cell of the present method is preferably isolated from a mammal.  
5 More preferably, the mammal is a human. It is well understood in the art that studies using hematopoietic stem cells of avian or murine origin are applicable to human studies. In clinical studies, especially in the field of hematology, the mouse is the preferred system when human donors are unavailable. Thus, it is understood by those skilled in the art that methods of using avian and murine hematopoietic stem cells can  
10 be readily adapted to uses involving human hematopoietic stem cells.

The step of contacting stem cells with a growth factor or a combination of growth factors is preferably *in vitro*. Thus, the stem cell is isolated, cultured, and contacted *in vitro* with a growth factor or combination of growth factors. The culture  
15 conditions can include a semisolid culture medium, but is not limited to that type of culture. By "growth factor" is meant an agent that, at least, promotes cell growth or induces phenotypic changes. In one embodiment of the invention, the growth factor or combination of growth factors of the present invention at least induces proliferation of hematopoietic stem cells or induces hematopoietic stem cells to differentiate into  
20 cardiomyocytes. The growth factor or combination of growth factors used in the present method of differentiating cardiomyocytes is selected from the group consisting of retinoic acid, stem cell factor, basic fibroblast growth factor, acidic fibroblast growth factor, endothelial cell growth factor, fibroblast growth factor-4, endothelin-1, interleukin (including, e.g., IL-2, IL-10 and IL-15), transforming growth factor alpha  
25 (TGF $\alpha$ ), transforming growth factor beta (TGF $\beta$ ), GM-CSF, IGF-1, platelet derived growth factor (PDGF), bone morphogenic factor-4, and Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11). Additionally, the hematopoietic stem can be contacted with dexamethasone, hyaluronic acid, 3,3',5-triiodo-L-thyronine, cAMP, angiotensin II, mannose,  $\beta$ -mercaptoethanol, and/or a Protein Kinase C (PKC) inhibitor

(including, e.g., sphingosine) to optimize cardiomyocyte differentiation. Endothelial cell growth factor comprises acidic fibroblast growth factor and heparin. Thus, it is understood that either endothelial cell growth factor or acidic fibroblast growth factor can be used interchangeably in the invention. Preferably, the combination of growth factors used in the present method of differentiating cardiomyocytes will comprise retinoic acid, basic fibroblast growth factor, fibroblast growth factor-4, Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11), bone morphogenic protein-4, and either acidic fibroblast growth factor or endothelial cell growth factor. Even more preferably, the hematopoietic stem can be additionally contacted with dexamethasone to optimize cardiomyocyte differentiation.

In one embodiment of the invention, the growth factor or combination of growth factors of the present invention at least induces proliferation of hematopoietic stem cells or induces hematopoietic stem cells to differentiate into skeletal muscle cells. The growth factor or combination of growth factors used in the present method of differentiating skeletal muscle cells is selected from the group consisting of retinoic acid, basic fibroblast growth factor, interleukin (e.g., IL-2 and/or IL-15), stem cell factor, IGF-1, endothelial cell growth factor, platelet derived growth factor, angiotensin II, TGF $\beta$ , and endothelin. Additionally, the hematopoietic stem can be contacted with dexamethasone, 3,3',5-triiodo-L-thyronine, cAMP,  $\beta$ -mercaptoethanol, and/or hyaluronic acid to optimize skeletal muscle cell differentiation. Preferably, the combination of growth factors used in the present method of differentiating skeletal muscle cells will comprise basic fibroblast growth factor, SCF, TGF- $\beta$  (preferably, TGF- $\beta$ 1), and endothelin.

25

A further inducing step of the present method can be used to induce the noncontracting cardiomyocytes derived from the stem cells to contract, wherein the inducement is by contacting the noncontracting cardiomyocytes with embryonic, fetal, or postnatal contracting cardiomyocytes; by changes in ion concentration (e.g., high

K<sup>+</sup>); by electrical or mechanical stimulation. The step of contacting either the cardiomyocytes, derived from the stem cells, can take place *in vivo* (e.g., by transplantation) or *in vitro*.

5           The preferred concentration of retinoic acid in the culture medium as used in the present invention is between  $1 \times 10^{-6}$  and  $1 \times 10^{-10}$  M. More preferably the concentration is between  $1 \times 10^{-7}$  and  $1 \times 10^{-9}$  M. Even more preferably, the concentration of retinoic acid is  $2 \times 10^{-8}$  M.

10           The preferred concentration of stem cell factor in the culture medium as used in the present invention is between 5 and 200 ng/ml. More preferably, the concentration is between 50 and 150 ng/ml. Even more preferably, the concentration is 100 ng/ml.

            The preferred concentration of basic fibroblast growth factor in the culture  
15   medium as used in the present invention is between 10 and 300 ng/ml. More preferably, the concentration is between 25 and 250 ng/ml. Even more preferably, the concentration is 100 ng/ml.

            The preferred concentration of acidic fibroblast growth factor in the culture  
20   medium as used in the present invention is between 10 and 300 ng/ml. More preferably, the concentration is between 25 and 250 ng/ml. Even more preferably, the concentration is 100 ng/ml.

            The preferred concentration of endothelial cell growth factor in the culture  
25   medium as used in the present invention is between 0.1 and 1000  $\mu$ g/ml. More preferably, the concentration is between 1 and 100  $\mu$ g/ml. Even more preferably, the concentration is 5  $\mu$ g/ml for the differentiation into cardiomyocytes, and 100  $\mu$ g/ml for the differentiation into skeletal muscle cells.

The preferred concentration of fibroblast growth factor-4 in the culture medium as used in the present invention is between 10 and 300 ng/ml. More preferably, the concentration is between 25 and 200 ng/ml. Even more preferably, the concentration is 100ng/ml.

5

The preferred concentration of endothelin-1 in the culture medium as used in the present invention is between  $1 \times 10^{-5}$  and  $1 \times 10^{-11}$  M. More preferably the concentration is between  $1 \times 10^{-6}$  and  $1 \times 10^{-10}$  M. Even more preferably, the concentration is  $1 \times 10^{-7}$  M.

10

The transforming growth factor- $\beta$  of the present invention includes transforming growth factor- $\beta$ 1, transforming growth factor- $\beta$ 2, and transforming growth factor- $\beta$ 3. The preferred concentration of transforming growth factor- $\beta$ 1, - $\beta$ 2, or - $\beta$ 3 in the culture medium as used in the present invention is between 0.1 and 100 ng/ml. More preferably, the concentration is between 1 and 50 ng/ml. Even more preferably, the concentration is 10ng/ml.

15

The preferred concentration of interleukin in the culture medium of the present invention is between 1 and 500 ng/ml. The preferred concentration of IL-10 is 25-75 ng/ml. Even more preferably, the concentration of IL-10 is 50 ng/ml. The preferred concentrations of IL-2 and IL-15 are 1-20 ng/ml, and, more preferably, 10 ng/ml.

20

The preferred concentration of transforming growth factor alpha ( $\text{TGF}\alpha$ ) in the culture medium of the present invention is between 0.5 and 50 ng/ml. More preferably the concentration is between 1 and 10 ng/ml, and, even more preferably, the concentration is 5 ng/ml.

25

As used in the present invention, the culture medium can comprise about 5-80% Wnt conditioned medium. Preferably, the culture medium comprises about 25-60%

Wnt conditioned medium. Even more preferably, the culture medium comprises about 50% Wnt conditioned medium.

5 The preferred concentration of dexamethasone in the culture medium of the present invention is between  $5 \times 10^{-6}$  and  $5 \times 10^{-12}$ . More preferably, the concentration is between  $1 \times 10^{-7}$  and  $1 \times 10^{-10}$ , and, even more preferably, the concentration is  $5 \times 10^{-7}$ .

10 The preferred concentration for a PKC inhibitor in the culture medium of the present invention is between  $2.5 \times 10^{-6}$  and  $2.5 \times 10^{-12}$  M. The concentration will vary depending on the PKC inhibitor that is used. For example, the preferred concentration of sphingosine is between  $1 \times 10^{-7}$  M and  $5 \times 10^{-7}$  M, and more preferably is  $2.5 \times 10^{-7}$  M.

15 The preferred concentration of platelet derived growth factor in the culture medium of the present invention is between 0.1 ng/ml and 100 ng/ml. The more preferred concentration is 1 ng/ml to 20 ng/ml. Even more preferably, the concentration is 10 ng/ml.

20 The preferred concentration of 3,3',5-triiodo-L-thyronine in the culture medium as used in the present invention is 2 ng/ml to 500 ng/ml. More preferably, the concentration is 10-100 ng/ml, and even more preferably the concentration is 20 ng/ml as used in promoting differentiation of cardiomyocytes, and 50 ng/ml as used in promoting differentiation of skeletal muscle cells.

25

The preferred concentration of cAMP in the culture medium as used in the present invention is  $5 \times 10^{-5}$  M to  $5 \times 10^{-3}$  M. More preferably, the concentration is  $1 \times 10^{-4}$  M to  $1 \times 10^{-3}$  M, and even more preferably the concentration is  $5 \times 10^{-4}$  M.



The preferred concentration of angiotensin II in the culture medium as used in the present invention is  $1 \times 10^{-8}$  to  $1 \times 10^{-6}$ . More preferably, the concentration is  $1 \times 10^{-7}$ M.

- 5        The preferred concentration of hyaluronic acid in the culture medium as used in the present invention is 10 µg/ml to 1000 µg/ml. More preferably, the concentration is 50 µg/ml to 500 µg/ml, and even more preferably the concentration is 100 µg/ml.

- 10       The preferred concentration of 2-ME (β-mercaptoethanol) in the culture medium as used in the present invention is 5 µM to 500 µM. More preferably, the concentration is 25 µM to 100 µM, and even more preferably the concentration is 50 µM.

- 15       The preferred concentration of GM-CSF in the culture medium as used in the present invention is 1 ng/ml to 1000 ng/ml. More preferably, the concentration is 50 ng/ml to 200 ng/ml, and even more preferably the concentration is 100 ng/ml.

- 20       The preferred concentration of IGF-1 in the culture medium as used in the present invention is 5 ng/ml to 500 ng/ml. More preferably, the concentration is 25 ng/ml to 100 ng/ml, and even more preferably the concentration is 50 ng/ml.

- 25       The preferred concentration of mannose in the culture medium as used in the present invention is 50 µg/ml to 5000 µg/ml. More preferably, the concentration is 250 µg/ml to 1000 µg/ml, and even more preferably the concentration is 500 µg/ml.

The preferred concentration of bone morphogenic factor-4 (or bone morphogenic protein-4 or BMP-4) in the culture medium as used in the present invention is 5 ng/ml to 500 ng/ml. More preferably, the concentration is 25 ng/ml to 100 ng/ml, and even more preferably the concentration is 50 ng/ml.

The culture medium used in the present invention preferably further comprises Iscove's Modified Delbecco's Medium, or the equivalent thereof, and 20% serum. Preferably the serum is fetal bovine serum.

5       The present invention also provides a process of promoting differentiation of a hematopoietic stem cell into a cardiomyocyte or skeletal muscle cell, comprising the steps of obtaining a hematopoietic stem cell with cardiomyocyte or skeletal muscle cell potential from an embryonic donor or nonembryonic donor and contacting the hematopoietic stem cell with a growth factor or combination of growth factors, thereby  
10       promoting differentiation of the hematopoietic stem cell into the cardiomyocyte or skeletal muscle cell. As an alternative embodiment, the hematopoietic stem cell can be contacted with cardiac tissue to promote differentiation of a hematopoietic stem cell into a cardiomyocyte or, alternatively, contacted with skeletal muscle to promote differentiation of a hematopoietic stem cell into a skeletal muscle cell. Hematopoietic  
15       stem cells can be isolated from the aorta-gonad-mesonephros area, the liver, the spleen, the yolk sac, or the bone marrow of an embryonic donor or from the liver, spleen, or, more preferably, the blood or bone marrow of a donor. The term "hematopoietic stem cell" does not include the totipotent embryonic stem cell. The present invention also provides an enriched population of hematopoietic stem cells with cardiomyocyte or  
20       skeletal muscle cell potential, wherein the hematopoietic stem cells are derived from an embryo.

The present invention provides a further process of promoting differentiation of a stem cell into a cardiomyocyte or skeletal muscle cell, comprising the steps of  
25       obtaining a stem cell with cardiomyocyte with skeletal muscle cell potential from a nonembryonic donor or a hematopoietic stem cells from a embryonic donor and transfecting the cell with a nucleic acid sequence that promotes differentiation of the stem cell into the cardiomyocyte or skeletal muscle cell. The transfecting step is performed using standard techniques well known in the art. (*Molecular Cloning*, eds.

Sambrook, Fritsch, and Maniatis, 1989). Examples of nucleic acid sequences that promote differentiation of stem cells into cardiomyocytes include, among other, the cardiac Nk2-type homeobox genes (e.g., Nkx2.3, Nkx2.5, Nkx2.6, Nkx 2.7, Nkx2.8 and Nkx2.9) (Patterson et al., 1998), its vertebrate ancestral gene *tinman* (Bodmer, 5 1993); GATA-4, GATA-5 and GATA-6 (Jiang et al., 1998), or serum response factor (SRF; Chen Swartz, 1996).

The present invention provides a further process of promoting differentiation of a hematopoietic stem cell into a cardiomyocyte or skeletal muscle cell, comprising the 10 steps of obtaining a hematopoietic stem cell with cardiomyocyte or skeletal muscle cell potential from an embryonic or nonembryonic donor and inducing the hematopoietic stem cells to differentiate into a contracting cardiomyocyte or skeletal muscle cell. Thus, the hematopoietic stem cell is induced to differentiate into a cardiomyocyte or skeletal muscle cell and further induced to become a contracting cardiomyocyte or 15 skeletal muscle cell *in vivo* without the step of transfecting the hematopoietic stem cell. Preferably, the induction step of promoting contraction is without contacting the differentiated cardiomyocyte or skeletal muscle cell with a growth factor or combination of growth factors.

20 Also provided herein is a population of cardiomyocytes or skeletal muscle cells derived by the process of the invention. The cardiomyocytes and skeletal muscle cells of the invention can be readily identified by their expression of at least one characteristic of a cardiomyocyte or a skeletal muscle cell, respectively. Specifically, the cardiomyocytes of the present invention are non-contracting but are capable of 25 contraction upon inducement. The skeletal muscle cells are non-twitching but are capable of twitching upon inducement. The cardiomyocytes or skeletal muscle cells of the present invention can be used in a variety of methods, such as transplantation. In addition, the cardiomyocytes or skeletal muscle cell can be used to screen pharmacological agents for their effect on cardiac or skeletal muscle activity,

comprising contacting a cardiomyocyte or skeletal muscle cell of the present invention with the compound and determining the effect of the compound on the activity of the cardiomyocyte or skeletal muscle cell. Thus, the present method further provides a method of screening for a compound that promotes cardiac or skeletal muscle activity

5 comprising stimulating the cardiomyocytes or skeletal muscle cells to contract or twitch, contacting the cardiomyocytes or skeletal muscle cells of the present invention with the compound, and determining whether the compound promotes cardiac or skeletal muscle activity. Effects on cardiac or skeletal muscle activity can be assessed using methods well known in the art, including, for example, assessing toxicity, protein

10 expression or secretion, contractile strength or frequency, and ion channel fluctuations (e.g., voltage dependent and receptor mediated  $Ca^{++}$  channel fluctuations).

The cardiomyocytes or skeletal muscle cells could also be used in an implantable artificial heart or ventricular assist device, for example, as the myocardial

15 layer. Thus, the cardiomyocytes or skeletal muscle cell can be used as the myocardial layer in a tissue-engineered heart or ventricular assist device. The cardiomyocytes and the skeletal muscle cells could be used also as a food source (including, for example, a source of protein for animal feed).

20 The present invention also provides a relatively pure population of stem cells with cardiomyocyte or skeletal muscle cell potential, wherein the stem cells are derived from a nonembryonic donor. Also provided is a relatively pure population of hematopoietic stem cells with cardiomyocyte or skeletal muscle cell potential, wherein the stem cells are derived from a nonembryonic or an embryonic donor. By "relatively

25 pure population" is meant a population of cells comprising at least about 80% stem cells with cardiomyocyte or skeletal muscle cell potential. More preferably, the population comprises at least about 90% stem cells with cardiomyocyte or skeletal muscle cell potential. Even more preferably, the population comprises at least about 95% stem cells with cardiomyocyte or skeletal muscle cell potential. Most preferably,

the population comprises at least about 99% stem cells with cardiomyocyte or skeletal muscle cell potential.

The stem cells of the invention can be used in a variety of methods, such as  
5 transplantation. Also, the stem cells can be used in a method of identifying nucleic acids with cardiomyocyte or skeletal muscle cell differentiation promoting activity, comprising transfecting a nucleic acid of unknown cardiac or skeletal muscle cell differentiation promoting activity into the stem cell of the invention, culturing the transfected cells, and determining the presence of differentiated cells having one or  
10 more characteristics of cardiomyocytes or skeletal muscle cells.

The present invention also provides a composition, comprising stem cells with cardiomyocyte potential derived from a nonembryonic donor and retinoic acid, stem cell factor, basic fibroblast growth factor, acidic fibroblast growth factor, endothelial  
15 cell growth factor, fibroblast growth factor-4, endothelin-1, interleukin (including, e.g., IL-2, IL-10 and IL-15), transforming growth factor alpha ( $TGF\alpha$ ), transforming growth factor beta ( $TGF\beta$ ), GM-CSF, IGF-1, platelet derived growth factor (PDGF), bone morphogenic factor-4, and Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11 ). Additionally, the composition can further comprise dexamethasone,  
20 hyaluronic acid, 3,3',5-triiodo-L-thyronine, cAMP, angiotensin II, mannose,  $\beta$ -mercaptoethanol, and/or a Protein Kinase C (PKC) inhibitor (including, e.g., sphingosine). In one embodiment, the composition comprises stem cells with cardiomyocyte potential derived from a nonembryonic donor and retinoic acid, basic fibroblast growth factor, fibroblast growth factor-4, Wnt (e.g., a member of the class  
25 Wnt5a, including e.g., Wnt11 ), bone morphogenic protein-4, dexamethasone, and either acidic fibroblast growth factor or endothelial cell growth factor. In another embodiment, the composition comprises stem cells with cardiomyocyte potential derived from a nonembryonic donor and retinoic acid, stem cell factor, fibroblast growth factor-4, endothelin-1, transforming growth factor beta, and either basic

fibroblast growth factor or endothelial cell growth factor in amounts to promote the differentiation of the stem cells into cardiomyocytes. The composition can alternatively comprise the stem cells and two or more of the factors, including, e.g., stem cells with cardiomyocyte potential derived from a nonembryonic donor, and retinoic acid and  
5 stem cell factor, retinoic acid and fibroblast growth factor-4, retinoic acid and endothelin-1, retinoic acid and transforming growth factor beta, retinoic acid and either basic fibroblast growth factor or endothelial cell growth factor, stem cell factor and fibroblast growth factor-4, stem cell factor and endothelin-1, stem cell factor and transforming growth factor beta, stem cell factor and either basic fibroblast growth  
10 factor or endothelial cell growth factor, fibroblast growth factor-4 and endothelin-1, fibroblast growth factor-4 and transforming growth factor beta, fibroblast growth factor-4 and either basic fibroblast growth factor or endothelial cell growth factor, transforming growth factor beta and either basic fibroblast growth factor or endothelial cell growth factor, or stem cell factor and fibroblast growth factor-4 in amounts to  
15 promote the differentiation of the stem cells into cardiomyocytes. Preferably, the stem cells are bone marrow or blood cells and, more preferably, hematopoietic stem cells, and, even more preferably, myeloid-lymphoid stem cells. The stem cells are preferably isolated from a donor that is a mammal and, more preferably, a human.

20 The present invention also provides a composition, comprising hematopoietic stem cells with cardiomyocyte potential derived from an embryonic or nonembryonic donor and retinoic acid, stem cell factor, basic fibroblast growth factor, acidic fibroblast growth factor, endothelial cell growth factor, fibroblast growth factor-4, endothelin-1, interleukin (including, e.g., IL-2, IL-10 and IL-15), transforming growth factor alpha  
25 (TGF $\alpha$ ), transforming growth factor beta (TGF $\beta$ ), GM-CSF, IGF-1, platelet derived growth factor (PDGF), bone morphogenic factor-4, and Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11). Additionally, the composition can further comprise dexamethasone, hyaluronic acid, 3,3',5-triiodo-L-thyronine, cAMP, angiotensin II, mannose,  $\beta$ -mercaptoethanol, and/or a Protein Kinase C (PKC) inhibitor

(including, e.g., sphingosine). In one embodiment, the composition comprises hematopoietic stem cells with cardiomyocyte potential derived from an embryonic or nonembryonic donor and retinoic acid, basic fibroblast growth factor, fibroblast growth factor-4, Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11), bone morphogenic protein-4, dexamethasone, and either acidic fibroblast growth factor or endothelial cell growth factor. In another embodiment, the composition comprises hematopoietic stem cells with cardiomyocyte potential derived from an embryonic or nonembryonic donor and retinoic acid, stem cell factor, fibroblast growth factor-4, endothelin-1, transforming growth factor beta, and either basic fibroblast growth factor or endothelial cell growth factor. The composition can alternatively comprise the hematopoietic stem cells and any combination of two or more of the factors, including, e.g., stem cells with cardiomyocyte potential derived from a nonembryonic donor, and retinoic acid and stem cell factor, retinoic acid and fibroblast growth factor-4, retinoic acid and endothelin-1, retinoic acid and transforming growth factor beta, retinoic acid and either basic fibroblast growth factor or endothelial cell growth factor, stem cell factor and fibroblast growth factor-4, stem cell factor and endothelin-1, stem cell factor and transforming growth factor beta, stem cell factor and either basic fibroblast growth factor or endothelial cell growth factor, fibroblast growth factor-4 and endothelin-1, fibroblast growth factor-4 and transforming growth factor beta, fibroblast growth factor-4 and either basic fibroblast growth factor or endothelial cell growth factor, transforming growth factor beta and either basic fibroblast growth factor or endothelial cell growth factor, or stem cell factor and fibroblast growth factor-4 in amounts to promote the differentiation of the stem cells into cardiomyocytes.

25       The present invention also provides a composition, comprising stem cells with skeletal muscle cell potential derived from a nonembryonic donor and retinoic acid, basic fibroblast growth factor, interleukin (e.g., IL-2 and/or IL-15), stem cell factor, IGF-1, endothelial cell growth factor, platelet derived growth factor, angiotensin II, TGF $\beta$ , and endothelin-1. Additionally, the composition can further comprise

dexamethasone, 3,3',5-triiodo-L-thyronine, cAMP,  $\beta$ -mercaptoethanol, and hyaluronic acid. In one embodiment, the composition comprises the stem cells with skeletal muscle cell potential derived from a nonembryonic donor and basic fibroblast growth factor, SCF, TGF- $\beta$  (preferably, TGF- $\beta$ 1), and endothelin. The composition can  
5 alternatively comprise the stem cells and any combination of two or more of the factors in amounts to promote the differentiation of the stem cells into skeletal muscle cells, including, e.g., retinoic acid and basic fibroblast growth factor, retinoic acid and interleukin, retinoic acid and PDGF, retinoic acid and angiotensin, retinoic acid and TGF $\beta$ , retinoic acid and endothelin, basic FGF and interleukin, basic FGF and SCF,  
10 basic FGF and IGF-1, basic FGF and ECGF, basic FGF and PDGF, basic FGF and angiotensin II, basic FGF and TGF $\beta$ , basic FGF and endothelin-1, SCF and interleukin, SCF and IGF-1, SCF and ECGF, SCF and PDGF, SCF and angiotensin II, SCF and TGF $\beta$ , SCF and endothelin-1, TGF- $\beta$  and interleukin, TGF- $\beta$  and IGF-1, TGF- $\beta$  and ECGF, TGF- $\beta$  and PDGF, TGF- $\beta$  and angiotensin II, TGF- $\beta$  and TGF $\beta$ , and TGF- $\beta$   
15 and endothelin-1. Preferably, the stem cells are bone marrow or blood cells and, more preferably, hematopoietic stem cells, and, even more preferably, myeloid-lymphoid stem cells. The stem cells are preferably isolated from a donor that is a mammal and, more preferably, a human.

20 The present invention also provides a composition, comprising hematopoietic stem cells with skeletal muscle potential derived from an embryonic or nonembryonic donor and retinoic acid, basic fibroblast growth factor, interleukin (e.g., IL-2 and/or IL-15), stem cell factor, IGF-1, endothelial cell growth factor, platelet derived growth factor, angiotensin II, TGF $\beta$ , and endothelin-1. Additionally, the composition can  
25 further comprise dexamethasone, 3,3',5-triiodo-L-thyronine, cAMP,  $\beta$ -mercaptoethanol, and hyaluronic acid. In one embodiment, the composition comprises hematopoietic stem cells with skeletal muscle potential derived from an embryonic or nonembryonic donor and basic fibroblast growth factor, SCF, TGF- $\beta$  (preferably, TGF- $\beta$ 1), and endothelin. The composition can alternatively comprise the hematopoietic stem cells



and any combination of two or more of the factors in amounts to promote the differentiation of the hematopoietic stem cells into skeletal muscle cells, including, e.g., retinoic acid and basic fibroblast growth factor, retinoic acid and interleukin, retinoic acid and PDGF, retinoic acid and angiotensin, retinoic acid and TGF $\beta$ , retinoic acid and endothelin, basic FGF and interleukin, basic FGF and SCF, basic FGF and IGF-1, basic FGF and ECGF, basic FGF and PDGF, basic FGF and angiotensin II, basic FGF and TGF $\beta$ , basic FGF and endothelin-1, SCF and interleukin, SCF and IGF-1, SCF and ECGF, SCF and PDGF, SCF and angiotensin II, SCF and TGF $\beta$ , SCF and endothelin-1, TGF- $\beta$  and interleukin, TGF- $\beta$  and IGF-1, TGF- $\beta$  and ECGF, TGF- $\beta$  and PDGF, TGF- $\beta$  and angiotensin II, TGF- $\beta$  and TGF $\beta$ , and TGF- $\beta$  and endothelin-1.

The present invention also provides a method of determining whether nonembryonic stem cells or embryonic hematopoietic stem cells of unknown cardiomyocyte or skeletal muscle potential can be promoted to differentiate into cardiomyocytes or skeletal muscle, comprising the steps of contacting the stem cells with a growth factor or combination of growth factors under conditions that allow the cells to differentiate, and determining the presence of differentiated cells having one or more characteristics of cardiomyocytes or skeletal muscle cells. The contacting step preferably occurs *in vitro*. For example, the stem cells can be cultured in semisolid or other type of culture medium. Thus, the cells can be allowed to differentiate in culture and screened for expression of cardiomyocyte- or skeletal muscle cell- specific proteins to determine whether the cells become cardiomyocytes or skeletal muscle cells. In one embodiment, the growth factor or combination of growth factors is selected from the group consisting of retinoic acid, stem cell factor, basic fibroblast growth factor, acidic fibroblast growth factor, endothelial cell growth factor, fibroblast growth factor-4, endothelin-1, interleukin (including, e.g., IL-2, IL-10 and IL-15), transforming growth factor alpha (TGF $\alpha$ ), transforming growth factor beta (TGF $\beta$ ), GM-CSF, IGF-1, platelet derived growth factor (PDGF), bone morphogenic factor-4, and Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11). Additionally, the cell to be screen can be

further contacted with dexamethasone, hyaluronic acid, 3,3',5-triiodo-L-thyronine, cAMP, angiotensin II, mannose,  $\beta$ -mercaptoethanol, and/or a Protein Kinase C (PKC) inhibitor (including, e.g., sphingosine). Preferably, the combination of growth factors used in the present screening method will comprise retinoic acid, basic fibroblast growth factor, fibroblast growth factor-4, Wnt (e.g., a member of the class Wnt5a, including e.g., Wnt11), bone morphogenic protein-4, and either acidic fibroblast growth factor or endothelial cell growth factor. Even more preferably, the cell to be screened can be additionally contacted with dexamethasone.

10 In another embodiment, the growth factor or combination of growth factors is selected from the group consisting of retinoic acid, basic fibroblast growth factor, interleukin (e.g., IL-2 and/or IL-15), stem cell factor, IGF-1, endothelial cell growth factor, platelet derived growth factor, angiotensin II, TGF $\beta$ , and endothelin. Additionally, the cell to be screened can be further contacted with dexamethasone, 15 3,3',5-triiodo-L-thyronine, cAMP,  $\beta$ -mercaptoethanol, and/or hyaluronic acid. Preferably, the cell to be screened will be contacted with basic fibroblast growth factor, SCF, TGF- $\beta$  (preferably, TGF- $\beta$ 1), and endothelin. The cells identified as being capable of being promoted to differentiate into cardiomyocytes or skeletal muscle cells can be made to differentiate and can be used in the treatment and screening methods of the 20 invention.

The invention also provides a method of treating a subject with heart injury or disease, comprising transplanting the cardiomyocytes of the present invention, or alternatively the stem cells with cardiomyocyte potential, into the cardiac muscle of the subject, thereby improving cardiac function. The invention also provides a method of 25 treating a subject with a muscle injury or disease, comprising transplanting the skeletal muscle cells of the present invention, or alternatively the stem cells with skeletal muscle potential, into the skeletal muscle of the subject, thereby improving muscle function. The subject can be an embryo, a fetus, or a postnatal subject. In one embodiment of the

invention, the cardiomyocytes, skeletal muscle cells, or stem cells are transfected prior to transplantation with a nucleic acid. The nucleic acid can comprise a nonmutated gene, wherein the subject being treated has a mutated gene. In either embodiment, the stem cells are preferably bone marrow or blood cells, and, more preferably,

5 hematopoietic stem cells, and even more preferably, myeloid-lymphoid stem cells.

In one embodiment of the invention, the cardiomyocytes or skeletal muscle cells of the present invention are transplanted. In an alternative embodiment, the stem cells of the present invention can be transplanted directly into cardiac or skeletal muscle tissue and be allowed to differentiate into cardiomyocytes or skeletal muscle cells. Using the

10 treatment method of the invention, the transplanted cardiomyocytes, skeletal muscle cells, or stem cells, can become integrated into the cardiac or skeletal muscle of the subject being treated. Ideally, the donor of the stem cells is also the subject being treated, thereby avoiding the need for chronic immunosuppression therapy.

Alternatively, the donor of the stem cells can be the mother of the developing

15 embryonic or fetal subject. Preferably, the subject being treated is a mammal and, more preferably, a human. Improvement in cardiac or skeletal muscle function of a subject following transplantation can be determined using a variety of clinical methods well known to those skilled in the art, including, for example, measuring cardiac output, blood pressure, and heart rate for improved cardiac function and increased contractility

20 or strength for skeletal muscle function.

As used in the specification and in the claims, "a" can mean one or more, depending upon the context in which it is used.

25 The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

## EXAMPLES

### Example 1

Using an embryonic mouse *ex ovo* culture system (Eto and Osumi-Yamashita, 1995; New, 1971; New, 1978; Sturm and Tam, 1993), retrovirally  $\beta$ -galactosidase labeled QCE-6 cells were microinjected into ED (embryonic day) 7.0-11.5 mouse embryos, which were then allowed to develop for 24-48 hrs. Within these mouse embryos, microinjected QCE-6 cells were able to incorporate into heart tissue and subsequently, differentiate into myocardial cells, as evidenced by immunoreactivity for the MF20 antibody. These results demonstrated that early nondifferentiated mesoderm cells can be transplanted into more developed host tissue and that nondifferentiated mesoderm stem cells possessing cardiac potential will incorporate into cardiac tissue and develop as cardiac cells when transplanted into living embryos.

### 15 Example 2

To determine if bone marrow stem cells possess the potential to manifest a cardiac phenotype, bone marrow cultures were established from the femurs of ED16 chicken or ED14 quail embryos as previously described (Coll and Ingram, 1978; Cormier and Dieterlen-Lièvre, 1990; Nicolas-Bolnet *et al.*, 1995). Bone marrow cells were segregated into distinct hematopoietic stem cell (HSC) and stromal cell populations following Ficoll-Paque separation and adhesion selection (Metcalf, 1984; Petzer *et al.*, 1996). These two types of primary bone marrow cultures were treated with the following factors, which were obtained from Sigma: retinoic acid ( $2 \times 10^{-8}$ ), stem cell factor (100 ng/ml), bFGF (100ng/ml), aFGF(100ng/ml), FGF-4 (100ng/ml), endothelin-1 ( $1 \times 10^{-7}$ ), TGF $\beta$  (10 ng/ml). Endothelial cell growth factor (5 $\mu$ g/ml) can be used instead of aFGF. Either TGF $\beta$ -1, -2, or -3 can be used. Cultures were stained two days later with either MF20 antibody, which recognizes both skeletal and heart muscle isoforms of sarcomeric myosin heavy chain, or the 109-19 antibody which

reacts specifically to a cardiac specific sarcomeric myosin isoform (Bader *et al.*, 1982; De Groot *et al.*, 1987). Both HSC and stromal cell cultures contained cells that labeled with the MF20 antibody, however, only the HSC cultures contained cells that expressed cardiac specific myosin. In other words, sarcomeric myosin positive cells derived from  
5 the HSC cultures expressed a cardiomyocyte phenotype, while those derived from the stromal cell cultures exhibited skeletal muscle phenotypes. Interestingly, the morphology of the sarcomeric myosin positive HSCs changed with this treatment. These cells were now adherent and also expressed muscle-specific  $\alpha$ -sarcomeric actin. Moreover, these cells did not exhibit the typical cell morphology associated with  
10 skeletal muscle cells but rather the cell shape characteristic of a cultured cardiomyocyte.

To further identify within the HSC population the cell type that displayed this cardiac protein, the expression patterns of certain proteins by the cells in bone marrow cultures were analyzed. Although each individual protein is not unique to early  
15 mesoderm, the coexpression of cytokeratin, vimentin, cingulin, N-cadherin and the  $\beta$ 1 integrin is particular to this tissue among cells of the early embryo. Immunostaining of nontreated HSC cultures showed a small number of cells that coexpressed these various proteins. These cells likewise showed positive reaction to the 2G6 antibody. This monoclonal antibody was prepared from mice immunized with the cell membrane  
20 fraction of nontreated QCE-6 cells. This antibody stains cardiomyocytes, endothelial, and red blood cells in both avian and murine tissue. In contrast, only vimentin and N-cadherin proteins were exhibited by the stromal cell population. In other words, stromal cell cultures did not exhibit any cells that demonstrated a phenotype typical of early mesoderm. Thus, bone marrow cells that exhibited an early mesodermal phenotype  
25 were found among the HSC population.

To determine if these early mesoderm-like bone marrow cells could manifest a contractile phenotype, these cells were incorporated into the avian blastoderm suspension cultures previously described (Eisenberg and Markwald, 1997). In these

experiments, either chicken or quail HSCs were labeled with a vital dye prior to mixing with blastoderm cells from HH stage 5 chicken or quail embryos. The results were quite exciting. Some of the HSCs were found incorporated into areas that demonstrated contraction prior to immunohistochemical staining. These cells were found to be sarcomeric myosin-positive, as were the blastoderm cells associated with them. To determine if these bone marrow derived cells had undergone myofibrillogenesis, bone marrow/blastoderm cell suspensions were plated at high micromass cell densities into 8-well Lab-Tek chamber slides. Following 4 days in culture, cells were stained with 109-19 antibody which specifically binds to cardiomyocytes. These bone marrow cells displayed myofibrils of cardiac-specific sarcomeric myosin. Additionally, these experiments were performed in parallel using bone marrow stromal cells. When mixed with HH stage 5 blastoderm cells, stromal cells did display a skeletal muscle phenotype. However, no cardiac phenotype, as determined by contractility and cardiac-specific protein expression, was observed in these stromal cell containing cultures.

15

These studies are the first to show that a population of bone marrow stem cells have the capacity to differentiate into and function as cardiac myocytes when associated with other cardiac cells.

### 20 Example 3

To determine whether adult bone marrow cells have cardiomyogenic potential, bone marrow cells were harvested from adult mice, Ficoll-Paque and adhesion separated, and cultured in conditions that stimulate cardiac differentiation *in vitro*. Adult CD1 or C57 male mice were sacrificed and the limbs removed. The bones of each limb were placed in  $\alpha$ MEM + P/S cell medium. The bones were cleaned of as much excess tissue as possible, then the tips of the bones were removed so that the bone marrow could be easily removed. The bone marrow was harvested from the bone by flushing the bone marrow out of the bone with a stream of cell medium from a 26G needle on a

1 cc syringe. The cells were pipetted into bacterial grade (nonadhering) petri dishes containing  $\alpha$ MEM cell medium. The cords of bone marrow were flushed apart by drawing the tissue up into a 10cc syringe with a 20G needle. Residual cells were washed from the surface of the plate and drawn into the 10cc syringe. The resulting cell suspension was run through a sterile sieve (70 $\mu$ m) into a 50ml conical tube to remove any large pieces of bone that remain. Ten-twenty ml of cell medium was run through the sieve to wash any remaining cells off of the sieve and into the centrifuge tube. The cells were spun in a centrifuge at 1,000RPM for 10 min. The cells were resuspended in 1ml  $\alpha$ MEM and a cell count, using the vital dye 4% trypan blue, with a hemocytometer (counting chamber), yielding the total number of cells extracted from the bone marrow. The cell sample was diluted to  $5 \times 10^6$  cells/ml, and 4ml of the diluted cells was layered atop 3ml of Ficoll/Paque, according to the manufacturer's instructions, in a 15ml conical centrifuge tube. The tubes were placed in the centrifuge and spun for 30min at 1,350 RPM. Subsequently, the band of leukocytes (i.e., the buffy coat) is harvested at the Ficoll/Pacque- $\alpha$ MEM interface. The resulting cells were diluted in 1ml  $\alpha$ MEM and counted. The cells were diluted to  $1 \times 10^6$  cells/ml in  $\alpha$ MEM containing 10% FBS and placed in a tissue culture dish (35mm, 60mm, or 100mm using 2ml, 5ml, or 10ml of cells, respectively). The cultured cells were incubated overnight at 5% CO<sub>2</sub> and 37°C for adherence depletion. The nonadherent cells were subsequently removed from each dish with a pipette and placed in a 15ml conical centrifuge tube. The dish was gently rinsed with  $\alpha$ MEM without serum to remove any remaining nonadherent cells and to remove serum from the dish, as serum interferes with the activity of trypsin. The cells were added to the tube of nonadherent cells. For use as controls, the adherent cells were loosened with trypsin-EDTA for approximately 10 min at 37°C. The adherent cells were removed as thoroughly as possible by gently pipetting and rapping the dishes against a hard surface, and the adherent cells were placed in centrifuge tubes. The tubes of adherent cells and the tubes of nonadherent cells were centrifuged for 10 min. The adherent and nonadherent cells were then resuspended in 1ml of  $\alpha$ MEM cell medium and counted.

To verify that the derived bone marrow cells are hematopoietic stem cells, a portion of the isolated cells were then plated in a semisolid culture medium. The following were placed in 12X75mm culture tubes: 0.45ml of fetal bovine serum (Sigma), 0.15ml of bovine serum albumin (Integen), 0.15ml of giant tumor cell  
5 conditioned medium (Origen), 1.5 $\mu$ l of erythropoietin (1 unit/ml), 1.5 $\mu$ l of stem cell factor (100ng/ml), 7.5 $\mu$ l  $\beta$ -mercaptoethanol ( $1 \times 10^{-4}$ /ml). The cells were added to the tubes at  $5 \times 10^4$ /ml and  $\alpha$ MEM was added to bring the total volume to 1.5ml per tube. Subsequently, 0.6ml of 3% methylcellulose was added to each tube. The tubes were then mixed by inversion, end over end, until the cultures were homogeneous. The  
10 semisolid culture was then drawn up into a 3cc syringe with a 16G needle, and squirted into tissue culture suspension dishes (35mm). Pairs of the suspension dishes were placed in a 20X100mm tissue culture dish, along with a 35mm dish filled with distilled water (to prevent the gels from drying out). The dishes were then placed in an incubator at 5% CO<sub>2</sub> and 37°C and incubated for 4-10 days and markers for HSCs were  
15 used to verify that the cells were HSCs.

The remaining cultured cells were then stained with either sarcomeric  $\alpha$ -actin or 492-4A5 antibody, the latter which binds specifically to murine cardiac myosin heavy chain. Both stromal cell- and HSC-derived cultures contained cells that exhibited  
20 sarcomeric protein expression, but only HSC-derived cells, in small numbers, exhibited reactivity to cardiac specific myosin antibodies. These results show that mammalian adult bone marrow contains cells, specifically HSC cells, that can express a cardiac phenotype under specific conditions. Moreover, adult bone marrow can be a source of mesoderm stem cells for transplantation.

25

As a further control, the cell populations were stained for the skeletal muscle-specific transcription factor MyoD. (Molkentin and Olson, 1996). Only stromal cells showed nuclear MyoD staining. These results indicate that sarcomeric protein expression of the HSC-enriched cells is indicative of cardiac muscle cells.



The percentage of cells within the isolated cultures of mammalian, nonadherent cells that manifested cardiac phenotype was 0.01%, as compared to 0.02% in avian cultures. The abundance of HSCs in adult mammals represents up to 0.05% of the cell population within bone marrow (Morrison *et al.*, 1995). These results, therefore, suggest that the cells with cardiomyocyte potential represent a subpopulation of the HSCs.

#### Example 4

Multipotent stem cells can be separated from lineage-committed progenitors based on their Lin<sup>-</sup> phenotype. Lin<sup>-</sup> designates the absence of lineage markers B220 (all pre-B and B cells), Gr-1 (granulocytes), Mac-1 (myelomonocytic cells) and CD4 and CD8 (T cells) (Spangrude *et al.*, 1988). A highly enriched population of Lin<sup>-</sup> multipotent stem cells can be obtained from the bone marrow using the murine-specific StemSep kit (StemCell Technologies, Inc.). Unlike Dynabeads, this is a negative selection technique where the cells of interest are purified from remaining unwanted cells targeted for removal by monoclonal antibodies and magnetic colloid. This method is very effective for instances when the desired cell type is rare. For isolation for multipotent hematopoietic stem cells from mammalian bone marrow, a cocktail of monoclonal antibodies specific for the lineage markers has proven successful (Spangrude *et al.*, 1988). The starting bone marrow cell suspension does not need to be subjected to Ficoll-Paque or adherence depletion. An antibody cocktail is simply added to the cell suspension followed by the addition of the magnetic colloid. The cell suspension is then passed through a magnetic column which binds the unwanted cells. Desired cells are collected in the flow through volume, ready to use. Like the Dynabeads, this technique is fast, far less costly than flow cytometry and does not affect the cells in any manner. To ascertain whether individual lineage committed progenitors from adult mouse bone marrow can differentiate into cardiomyocytes, StemSep with the

multiple lineage specific antibody cocktail, minus one or more of the antibody reagents, can be used.

- Alternatively, cell specific antibodies (e.g., Gr-1) in conjunction with
- 5 Dynabeads or CELlection Positive Isolation System (Dyna) can be used to positively select the HSC subpopulation to be tested. The CELlection Positive Isolation System reagent consists of superparamagnetic microspheres to which biotinylated antibodies are attached via a cleavable linker. Thus, the desired population is purified by magnetic separation and subsequently released from the particles for further experimentation.
- 10 Antibodies to be used include CD33 antibody for harvesting myeloid progenitors and CD9, CD10, and B220 for pre-B cells. These are biotin-labeled using EZ-Link biotin hydrazide (Pierce, Rockford, IL). Cells are incubated with biotin-antibodies on ice and subsequently washed in cold PBS/5% fetal bovine serum. The beads are mixed with this cell suspension at 4°C, forming a complex which is then isolated using a magnet.
- 15 The purified cells are dislodged from these spheres using a releasing buffer, supplied with the kit. All steps are done according to the manufacturer's instructions. This method yields cells that are pure, viable, and phenotypically unaltered.

- The cells isolated by either the StemSep or CELlection procedures can be
- 20 cultured as described in the previous Examples to stimulate cardiac differentiation and assayed for cardiac protein expression. Alternatively, the subpopulations of HSC cells can be added to individual wells of fibronectin-coated Lab-Tek chamber slides (Nunc, Naperville, IL) and treated for 48 hours with growth factor cocktail, as described above to stimulate cardiac differentiation of non-separated HSCs. The cultures can then be
- 25 assayed for cardiac protein expression using immunohistochemistry. The MF20, which recognizes all isoforms of sarcomeric myosin heavy chain, and the anti-murine cardiac specific myosin antibody 492-4A5, as well as the myosin light chain-2 (MLC-2a) atrial and ventricular (MLC-2v) antibodies, can be used to assay cultures for cardiac phenotypes (Gruber *et al.*, 1996; Kubalak *et al.*, 1994; O'Brien *et al.*, 1993). The latter

three antibodies recognize only cardiac tissue in mice. Thus, the cells with cardiomyocyte potential can be isolated.

HSCs cultured under various culture conditions known to stimulate specific lineages show that HSCs allowed to develop toward the myeloid-lymphoid stem cell intermediate retained the capacity to express muscle associated proteins. Thus, monoDC cell clusters, aggregates that represent a subset of cells restricted in lineage to monocytes, macrophages, and B, T, NK, and dendritic cells, were positive for sarcomeric myosin heavy chain following treatment with the growth factor cocktail that stimulates cardiac differentiation.

The cells with cardiac myocyte potential can be expanded according to the conditions as previously described (Yonemura *et al.*, 1997). Stem cells can be grown in medium containing  $\alpha$ -MEM (GibcoBRL), 20% (vol/vol) fetal calf serum (Hyclone), 1% deionized Fraction V bovine serum albumin (StemCell Technologies, Inc.),  $1 \times 10^{-4}$  mol/L 2-mercaptoethanol (Sigma), 100 ng/ml human recombinant FLT3/FLK-2 and 20 ng/ml human recombinant IL-II (both from PeproTech). Cells can be cultured in 5% CO<sub>2</sub>/95% air within suspension dishes (Nunc) and fed fresh medium every 7 days for up to 21 days. The rate of cell growth can be determined with a hemocytometer using trypan blue exclusion. The number of cell generations during the time interval can be calculated using the formula of  $g = (\log N - \log N_0) / \log 2$  where N is the number of cells at the end of the time interval and N<sub>0</sub> is the number of cells at the beginning (Eisenberg and Bader, 1996).

It is possible that cells committed to the GEMM (granulocyte, erythrocyte, megakaryocyte, monocyte) lineage still possess cardiac competence. Among these cells, the most likely cardiac competent cell is the myeloerythroid (GEMM) progenitor. To enrich for this cell type, we can perform two-step methylcellulose cultures (Hirayama and Ogawa, 1996; Hirayama *et al.*, 1992). HSCs can be isolated as

described above and plated in 35 mm suspension culture dishes (Nunc) containing  $\alpha$ -MEM (GibcoBRL), 1.2% (wt/vol) 1500 centipoise methylcellulose (StemCell Technologies, Inc.), 30% (vol/vol) fetal calf serum (Hyclone), 1% deionized Fraction V bovine serum albumin (StemCell Technologies, Inc.),  $1 \times 10^{-4}$  mol/L 2-mercaptoethanol (Sigma), 5% (vol/vol) conditioned medium from pokeweed mitogen-stimulated spleen cells (PWM-SCCM; StemCell Technologies, Inc.), 100 ng/ml murine stem cell factor (SCF), 100 ng/ml human recombinant FLT3/FLK-2, 20 ng/ml human recombinant IL-II, 100 ng/ml murine IL-6, and 100 ng/ml human recombinant (G-CSF) granulocyte colony-stimulating factor (all from PeproTech) in 5% CO<sub>2</sub>/95% air for 11 days. The primary colonies can then be individually lifted, washed in  $\alpha$ -MEM and replated in the above conditions plus the addition of 100 ng/ml murine recombinant IL-3 (PeproTech) for 2 more days. This method has been shown to promote the development of the GEMM progenitor while inhibiting the formation of the lymphocyte progenitors (Hirayama and Ogawa, 1996; Ogawa *et al.*, 1997). Verification of both HSC and GEMM progenitor cell enrichment can be done by cytological examination with May-Grunwald Giemsa as well as antibody selection as described above. For example, if the HSC expanded still retained that same phenotype, then these cells would pass through the StemSep column, yielding approximately the same number of cells as at the start of the selection. However, if during culture conditions these cells differentiated further down the hematopoietic lineage, they would be retained in this column, as demonstrated by a lower cell yield.

To test for the retention of cardiac potential following *in vitro* expansion of these cell subpopulations, cells can be cultured as described in the previous Examples and assayed for cardiac specific protein expression. The MF20, anti-murine cardiac specific myosin 492-4A5,  $\alpha$ -sarcomeric actin, cTnI, MLC-2a and MLC-2v antibodies can be used to assay cultures for cardiac phenotypes. Control cultures can consist of mouse fetal cardiomyocytes and adherence depleted bone marrow cells. The latter cell population can produce cells with cardiac phenotypes following growth factor

treatment. Additionally, the phenotype of expanded cell cultures in the absence of growth factors can be examined. Both nonadherent bone marrow cells and the *in vitro* expanded cultures can be examined by either immunolabeling with antibodies such as the common leukocyte antigen CD45, CD14 (monocyte) and the macrophage Mac-1 antigen CD11b/18 (Roche Molecular Biochemicals, Indianapolis, IN) and staining with May-Grunwald Giemsa dyes to determine if blood cell differentiation was provoked. The results of this phenotypic analysis guide further alterations in the expansion conditions in order to maintain the preferred stem cell state of the selected bone marrow cells. For example, if granulocytes were promoted by the *in vitro* expansion and this led to a noncardiogenic cell population, then G-CSF would be replaced in the chemically defined cocktail.

#### Example 5

The ability of bone marrow-derived cardiomyocytes to function as normal cardiac tissue can be determined using the embryonic mouse *ex ovo* culture system. Furthermore, mouse embryos can be allowed to grow *ex utero*, to analyze the functional ability of the bone marrow-derived cells over a longer period of time.

To prepare rat serum, methods known in the art can be used (Cockroft, 1990). For example, retired breeder male rats can be anesthetized with Halothane (Henry Schein) prior to exposure of the inferior vena cava. Approximately 12 ml of blood is collected and immediately centrifuged at 3500 rpm for two 5 min spins. The fibrin clot formed in the serum is squeezed with a sterile forceps to release the serum and the blood is then centrifuged again for 10 min. The clear serum is pooled from all samples and centrifuged again for 10 min to removed residual blood cells. The resulting serum is aliquoted together with 100 mg/ml streptomycin and 100 IU/ml penicillin into clean 50 ml conical tubes prior to storage at -20°C. Care is taken not to store the serum longer than 1 month. On the day of use, the serum is thawed at 37°C and heat

inactivated at 56°C for 35 min. Appropriate amount of Dulbecco's modified Eagle's medium (DMEM) can be added for a final volume of 3-4 ml of culture media per glass roller culture bottle. Media filled bottles are placed in the rotating drum under continuous gassing for at least 1 hour prior to culture of embryos (this allows for the  
5 equilibration of media with gas). The proportion of serum to media and the percentage O<sub>2</sub> is dependent upon the age of the embryo prior to culture. Younger embryos require a higher percentage serum, but less oxygen in the media, while the older embryos require less serum and more oxygen.

10 Whole mouse embryos can be cultured as previously described (Sturm and Tam, 1993). Timed pregnant female mice are sacrificed, and each uterus removed and placed into sterile, phosphate buffered saline (PBS) to rinse off residual blood. The uterus is then transferred to PB1 media (in mM: 137 NaCl, 2.7 KCl, 0.5 MgCl<sub>2</sub>, 8.04 Na<sub>2</sub>HPO<sub>4</sub>, 1.47 KH<sub>2</sub>PO<sub>4</sub>, 0.9 CaCl<sub>2</sub>, 0.33 Na pyruvate, 1g/L glucose, 0.01g phenol red, 100 mg/ml  
15 streptomycin, 100 U/ml penicillin, pH 7.35) for removal of embryos. The decidual mass and Riechert's membrane are removed leaving the visceral yolk sac, the amnion, and the ectoplacental cone intact with embryos ED7.5-10.5 of age. Older embryos between ED10.5-11.5 are dissected further to pull away the visceral yolk sac and amnion from the embryo, leaving them attached so as to not disrupt the vessels  
20 connecting the embryo to the yolk sac or the umbilical vessels from the embryo to the placenta. Following microinjection of bone marrow-derived cells, embryos are transferred to the pre-equilibrated media in the roller culture bottles and incubated for the following 12-72 hours. The total time that the embryos are in culture depends both on the age of the embryo prior to culture (younger embryos can be cultured for a longer  
25 time than older ones) and on the desired time period for a particular experiment.

Multipotent HSC obtained from either ED14 quail bone marrow cells subjected to QH1 and/or 2G6 plus TAC1 selection or adult mouse bone marrow cells are enriched using StemSep and the Lin<sup>+</sup> antibody cocktail. Prior to transplantation, cells are

labeled with a vital dye PKH26-GL (Sigma) as described (Eisenberg and Markwald, 1997). Unlike DiI, this dye is not leaky for it becomes incorporated into the plasma membrane where it can remain stable for up to 100 days in culture. Bone marrow cells label quite well with this dye. HSCs are injected using beveled-edged glass needles  
5 connected to a simple syringe mounted on a micromanipulator (World Precision Instruments). Depending on cell number and the age of the embryo, microinjection volumes range from 0.5-4.0  $\mu$ l. Lower volume amounts are used with younger embryos because of their fragility. Only a limited number of cells (1-12) can be transplanted directly into embryonic heart tissue. However, as many as 50 cells can be injected if  
10 delivery is through the yolk sac vessel. All microinjections are done with the aid of a dissecting microscope.

Cardiac phenotype can also be assessed after a longer period of time beyond that obtainable in *ex ovo* embryo cultures by using microinjections of embryos left *ex utero*.  
15 These experiments allow following the potential of these cells up until birth and beyond if desired. As with the *ex ovo* studies, all mice can be of the same allelic background, thus avoiding graft rejection. Because these cells can be in the embryo for a longer period of time, the cells are retrovirally labeled prior to transplantation. HSCs are easily transfected with retrovirus (Nienhuis *et al.*, 1997; Sekhar *et al.*, 1996). Specifically, the  
20 transfected cell contains both the bacterial  $\beta$ -galactosidase gene and green fluorescent protein (GFP). Therefore, purified cell populations obtained in the previous Examples are labeled as described previously (Eisenberg and Markwald, 1997). Briefly, approximately  $2 \times 10^6$  bone marrow cells are exposed for 30 minutes at 37°C to  $2-3 \times 10^7$  virions/ml of medium. Following three consecutive washes in medium, cells are  
25 plated in suspension cultures containing  $\alpha$ -MEM (GibcoBRL), 20% (vol/vol) fetal calf serum (Hyclone), 1% deionized Fraction V bovine serum albumin (StemCell Technologies, Inc.),  $1 \times 10^{-4}$  mol/L 2-mercaptoethanol (Sigma) overnight at 37°C in 5% CO<sub>2</sub>/95% air. Those cells retrovirally transfected are marked by the GFP and thus can

be separated from those nontransfected on a FACStarplus cell sorter (Becton Dickinson) the next day.

*Exo utero* culture of mouse embryos are performed as described previously (Muneoka *et al.*, 1986) with slight modifications (Gruber *et al.*, 1998). All microinjections can be performed on embryos ranging from ED10 through ED13, when it is possible to visualize the body through the extraembryonic membranes. Timed-pregnant female mice are anesthetized with an intraperitoneal injection of 0.2 ml/10g body weight of Avertin (consisting of 2.5g 2,2,2-tribromoethanol, 5 ml 2-methyl-  
10 butanol (Aldrich) and 195ml sterile dH<sub>2</sub>O). Both uterine horns are exposed using a midline abdominal incision. Access to the embryos can be accomplished by a longitudinal cut through the uterine myometrium. The myometrium is then pulled back to the base of the placenta, leaving the embryos, with extraembryonic membranes intact, exposed to the abdominal cavity. Embryos are washed copiously with sterile  
15 saline both before and after injection. The abdominal cavity is bathed in penicillin G 100U/ml + streptomycin 100µg/ml in sterile saline before the abdomen is closed with a running 7-0 silk suture. After surgery, the mother is kept at 37°C and monitored until she has recovered from surgery. The advantage of using Avertin as an anesthesia, is that the recovery time is shorter, usually 4 to 6 hrs.

20

At the termination of the culture periods, embryos are cryopreserved for immunohistochemistry as described (Gruber *et al.*, 1998; Kitten *et al.*, 1987). This procedure entails washing the embryos in PBS and fixing by perfusion in 4% buffered paraformaldehyde/PBS, prior to an incubation in the same fixative for 2 hr at 4°C.  
25 Following several PBS washes, the embryos are passed through a graded series of sucrose solutions prior to embedding in a 1:1 ratio of Aquamount/OTC (Miles) medium on a solid block of dry ice. Cryostat 10µm sections are cut and rinsed in PBS before being neutralized in 50 mM NH<sub>4</sub>Cl/PBS for 5 min. Tissue is permeabilized and blocked in PBT (1.0% Triton X-100/PBS), 3% BSA (Sigma) and 10% normal goat



serum for 25 min at room temperature. Sections are stained for one or more of the following: QH1, 2G6, TAC1, 109-19, 492-4A5, MF20,  $\alpha$ -sarcomeric actin, cTnI, MLC-2a, MLC-2v and  $\beta$ -galactosidase (5'3' Inc.) antibodies, depending upon species of the transplanted cells. All FITC-conjugated secondary antibodies are obtained from  
5 Jackson Immuno Research Labs, Inc. Slides are imaged as described (Eisenberg *et al.*, 1997) on a Bio-Rad MRC-600 confocal connected to a Zeiss Axiophot inverted microscope.

#### Example 6

10

Confluent cultures of bone marrow-derived cardiomyocytes, as described in the previous Examples, are harvested following trypsin/EDTA treatment to obtain a single cell suspension in sterile Dulbecco's phosphate buffered saline (DPBS). The suspension is injected into adult heart as described previously, with minor modifications  
15 (Rockman *et al.*, 1991). Specifically, eight to twelve-week old adult mice are anesthetized with an intraperitoneal injection of 0.2 ml/10g body weight of Avertin (consisting of 2.5g 2,2,2-tribromoethanol, 5 ml 2-methyl-butanol and 195ml sterile dH<sub>2</sub>O). Once anesthetized, the animal is intubated, with positive-pressure ventilation being maintained using a Harvard murine ventilator (model 687) set at a tidal volume of  
20 0.2 ml and respiratory rate of 110/min. The murine heart is exposed by a small incision in the second intercostal space at the left upper sternal border. With the aid of a dissecting scope, cells are injected directly into the ventricular myocardium through a 30-gauge double-beveled needle connected to a plastic syringe. Approximately  $1 \times 10^4$  to  $10 \times 10^4$  cells are delivered in a volume of 2 to 3  $\mu$ l. The chest cavity is then closed  
25 with a 7-0 nylon suture. Following extubation and evacuation of the pneumothorax, the mouse is kept at 37°C and monitored until it has recovered from surgery.

Cells grafted into adult hearts are examined for their protein expression as well as their ability to incorporate into the surrounding tissue. The phenotype exhibited by

these cells is determined by immunohistochemical analysis. The development of normal sarcomeric structure, intercalated discs and gap junctions is investigated further by electron microscopy. Special attention can be given to the formation of gap junctions between grafted and nongrafted cells, a sign of intercellular coupling and complete cell incorporation into the tissue. Adult murine hearts are harvested and cryopreserved. The 10µm cryostat sections can be double stained with the following antibodies: MF20, MLC-2a, MLC2v and β-galactosidase or GFP, the latter two depending on the gene label being expressed by these cells.

For transmission electron microscopy examination (TEM), adult hearts are be harvested, washed in buffer (0.1 mol/L sodium phosphate, pH 7.3) and fixed overnight in 2.5% glutaraldehyde (Eisenberg and Bader, 1996). For cells labeled with the GFP-β-galactosidase retrovirus, hearts are stained for β-gal activity as described (Eisenberg and Markwald, 1997). The product of this reaction is an electron-dense precipitate which can be detected with TEM. The tissue is vibratome sectioned as described (Eisenberg *et al.*, 1997) and the resulting 200µm slices is embedded into LX-112 resin (Eisenberg and Bader, 1996). Ultrathin 60 nm sections is examined under a JEOL electron microscope (JEOL, Inc.). For cells labeled with the GFP adenovirus, sections are stained with the GFP primary antibody followed by an immunogold labeled secondary antibody.

#### Example 7

The identified bone marrow cells with cardiac potential are early precursor cells. This was shown using a technique for isolating primitive hematopoietic cells. 5-Fluorouracil (5-FU) is a cell cycling drug that specifically eliminates dividing cells. Cells that are in G1 phase of the cell cycle incorporate 5-FU metabolites and thus die as they enter the S phase (Hodgson and Bradley, 1979). It is known that pluripotent HSCs are quiescent and thus resistant to the drug. Hence, treatment of bone marrow cultures selectively enriches for this cell type (Lerner and Harrison, 1990). Embryonic

day 14 (ED14) quail bone marrow cells were isolated. HSC-containing nonadherent and adherent (stromal) cell population were both treated with high doses (25  $\mu$ g/ml) of 5-FU for 24 hours. The cultures were then washed several times with PBS to remove the 5-FU. The cultures were then incubated in a medium containing retinoic acid, stem cell factor, bFGF, FGF-4, endothelin-1, TGF $\beta$ , and either aFGF or endothelial cell growth factor as described above to promote cardiomyocyte differentiation. The cultures were then fluorescently stained using antibodies to sarcomeric myosin heavy chain. The majority of the nonadherent cells and stromal cells plated were killed by the second day of treatment. Of the surviving cells in the stromal cultures, no cardiac phenotype was observed following treatment with the combination of growth factors described above. Virtually all of the pluripotent HSCs, the surviving cells from among the nonadherent cell cultures, differentiated into cardiomyocytes as evidenced by positive reactivity for sarcomeric myosin.

#### 15 Example 8

Nonadherent bone marrow stem cells were obtained from adult ICR mice (10 weeks old) without selecting for 5-FU resistance. The cells were labeled with the vital dye PKH26-GL as described above. Labeled HSC population were microinjected into the ventricles of ED9.0 ICR mouse embryos. Embryos were allowed to develop in *ex ovo* cultures as described above. Labeled adherent bone marrow cells were also injected as controls. Following 24 hours in culture, embryos were harvested, cryopreserved, and sectioned for immunohistochemistry. Even after only 24 hours in culture, some of the nonadherent HSCs incorporated into the ventricular tissue and began to express sarcomeric myosin. Those that were found outside the heart did not express the protein. Conversely, the adherent cells did not graft well into heart tissue and did not express the myocyte proteins. These results show that bone marrow cells can be grafted into heart tissue.

**Example 9**

To further characterize the differentiation potential of bone marrow cells, cardiac gene expression was analyzed using reverse-transcriptase polymerase chain reaction (RT-PCR). HSC-enriched bone marrow cells from ED14 quail were cultured for 2-4 days in the absence or presence of growth factor cocktail, as described above. RNA was then harvested and subjected to RT-PCR amplification, using techniques well known in the art. (See, e.g., Molecular Cloning, eds. Sambrook, Fritsch, and Maniatis, 1989). In the RT-PCR amplification, oligonucleotide primers for either of the cardiac transcription factors Nkx2.5 (5'-CCTTCCCCGGCCCCTACTAC-3') (SEQ ID NO.:1) or GATA4 (5'-CTCCTACTCCACCCCTTACC-3' (SEQ ID NO:2) and 5'GCCCTGTGCCATCTCTCCTC-3' (SEQ ID NO:3)) were used. (Durocher, *et al.*, 1997). Amplification of the housekeeping gene GAPDH was used as a control. The cardiac homeobox gene Nkx2.5 (Durocher, et al., 1997) was not expressed by non-treated HSC-enriched cells from quail bone marrow. Cultures treated with growth factors that promote cardiac differentiation, however, showed a substantial induction of Nkx2.5 expression, although amplification of GAPDH was comparable in control and treated cells. A similar result was obtained for GATA4, as growth factor treatment promoted GATA4 expression but GATA4 was not amplified from untreated cells.

20

**Example 10**

To expand the HSCs, nonadherent quail bone marrow cells were isolated as described above and then treated with a high dose of 5-fluorouracil (5-FU) as in Example 7. The cells were cultured subsequently in medium containing 100 ng/ml stem cell factor (SCF), 100 ng/ml FLT3/FLK2 and 20 ng/ml IL-11. The cells were then expanded for one week to ten days, resulting in approximately a 1000 fold increase in the number of HSC cells. Thus, approximately  $10^7$  bone marrow cells were obtained from a dozen quail embryos, and approximately 1000 cells survived the 5-FU treatment.

This cell yield increased to greater than  $10^6$  after 10 days of expansion. After these cells were treated with growth factors to promote cardiac differentiation as described above, 10-20% of the cells exhibited a cardiac phenotype as demonstrated by positive MF20 or sarcomeric myosin immunolabelling. Those cells that failed to exhibit a cardiac  
5 phenotype died after one week of treatment. These results indicate that enriched HSCs from bone marrow can be expanded and still retain cardiac potential.

#### Example 11

10 As described in Example 8, nonadherent bone marrow stem cells were isolated from adult mice (C57BL/6) and labeled with the vital dye PKH26-GL. Labeled HSC cells were microinjected into the left ventricles of 12-week old C57BL/6 mice. Two weeks later, the animals were sacrificed, and the hearts isolated, cryopreserved, and sectioned for immunohistochemistry. Nonadherent HSCs incorporated into the  
15 ventricular tissue, and some of the cells expressed the cardiac-specific marker, ventricular myosin light chain-2 (MLC2v). Thus, fluorescent dye labeled HSCs were incorporated into MLC2v-positive ventricular cardiomyocytes. Furthermore, a small number of vital dye labeled cells were also MLC2v positive. These results show that adult bone marrow cells can be engrafted successfully into adult heart tissue and that a  
20 population of the HSC cells undergo cardiac differentiation within the permissive environment.

#### Example 12

25 Embryonic mesodermal stem cells fully differentiate into cardiomyocytes upon co-culture with explants of cardiac tissue fragments from tubular heart stage embryos. Specifically, virally labeled QCE6 cells were co-cultured with a HH stage 16 quail ventricular tissue fragment. After 48 hours of incubation, the cultures were dual stained for both sarcomeric myosin and  $\beta$ -galactosidase (a marker for the viral label). A

population of the  $\beta$ -galactosidase reactive cells were also reactive for sarcomeric myosin. Only those QCE6 cells that integrated into the ventricular tissue underwent cardiac differentiation.

### 5 Example 13

Similar to the methods of Example 12 using embryonic mesodermal cells, bone marrow cells can be co-cultured with embryonic stem cells or embryonic heart tissue. For co-cultures with embryonic stem cells, the embryonic stem cell line WW6, which is  
10 of C57BL/6 background. To be able to determine the identity of the bone marrow derived cells, the cells are obtained from  $\beta$ -gal positive ROSA26 mice. Aggregates are produced using hanging drop cultures. (See Eisenberg and Markwald, 1997). Purified HSCs and ES cells are mixed at ratios of 1:9 to 1:19 and suspended in medium at concentrations of  $1.25$  to  $2.5 \times 10^6$  cells/ml and distributed in  $20\mu\text{l}$  drops on the lids of  
15 sterile, 100 mm Petri dishes. The lids are inverted onto the dishes, each containing 2ml of sterile PBS. Following 48 hours of incubation, cell aggregates are transferred to new Petri dishes containing medium and incubated for 4 additional days. These aggregates are routinely observed microscopically to determine the extent of cell differentiation (i.e., presence of beating cells). To examine individual cells, the aggregates are  
20 partially dissociated by gently pipeting after treatment with 0.25% trypsin for 10 min. The resulting cell clumps are suspended in medium and plated into chamber slides for 12 hours prior to staining for both  $\beta$ -gal and individual sarcomeric proteins. Reagents specific for  $\beta$ -gal are commercially available as either rabbit polyclonal (5Prime-3Prime) or mouse monoclonal (Sigma) antibodies, which can be used in conjunction  
25 with either the monoclonal MF20 or polyclonal 492-4A5 myosin antibodies, respectively. Secondary antibodies specific for rabbit or mouse primary reagents are labeled with either fluorescein or Cy5 to detect protein expression.

For co-cultures with embryonic heart tissue, ED 12 embryos are extracted from timed pregnant C57BL/6 mice and placed in PBS. The visceral yolk sac and amnion are removed, and the entire heart tube removed and placed in fresh PBS. The hearts are then microdissected to obtain tissue fragments from either atrial or ventricular regions, with great care made to ensure that individual explants are segment specific. The individual fragments are then slit along one side and placed endocardial side down in individual wells of fibronectin-coated chamber slides, at a density of 2-3 region-specific fragments per well. The segmental identity of the cultured tissue are verified by immunostaining for both MLC2a and MLC2v, which are atrial-specific and ventricular-specific proteins, respectively (O'Brien *et al.*, 1993; Kubalak *et al.*, 1994). Atrial and ventricular fragments are incubated for four hours to facilitate attachment to the fibronectin substrate, prior to the addition of  $10^5$  purified HSCs obtained from adult ROSA26 mouse bone marrow. Cultures are incubated at 37°C for up to four days and microscopically analyzed daily for contractility prior to fixation of the tissue. After fixation, the tissue are dual stained for both  $\beta$ -gal (to identify the bone marrow derived cells) and either MF20, MLC2a, or MLC2v. In parallel, purified HSCs are cultured alone, treated with cardiac inducing growth factors and stained for either MLC2a or MLC2v.

Cardiomyocytes derived from bone marrow cells co-cultured with either embryonic stem cells or embryonic heart tissue can also be examined using electron microscopy as described above in Example 6.

#### Example 14

25

To promote differentiation of hematopoietic cells to myeloid-lymphoid stem cell intermediate, culture conditions for mouse HSCs were modified. Specifically, the mouse HSCs were grown in Iscove's Modified Dulbecco's Medium (Gibco/BRL) with 5% fetal bovine serum (Sigma) + 50 $\mu$ M 2-ME ( $\beta$ -mercaptoethanol; Sigma) + 100 ng/ml

GM-CSF (peproTech) for six days at 37°C in 5% CO<sub>2</sub>. This treatment selectively expands this population, giving rise to typical, myeloid-lymphoid stem cell aggregates. These aggregates are loosely adherent to tissue culture plastic and therefore can easily be dislodged and harvested by gentle pipetting.

5

To promote cardiac differentiation, the aggregates were then transferred to a tissue culture dish containing Iscove's Modified Dulbecco's Medium (Gibco/BRL) + 20% fetal bovine serum (Sigma). Under these conditions, cells released from the aggregates attached to the plastic and exhibited a fibroblast morphology. Once this occurred, the cells were collected and plated into fibronectin-coated (Human plasma fibronectin at 10µg/cm<sup>3</sup>; Collaborative) tissue culture plastic vessels containing 50% Iscove's Modified Dulbecco's Medium (Gibco/BRL) + 50% Wnt11 (or, alternatively, any Wnt of the class Wnt 5a) conditioned medium + 20% fetal bovine serum (Sigma) + 50µM 2-ME + retinoic acid ( $2 \times 10^{-8}$  M; Sigma) + FGF-4 (100 ng/ml; Sigma) + bFGF (100 ng/ml; PeproTech) + IL-2 + IL-15 (both at 10 ng/ml; PeproTech) + IL-10 (50 ng/ml; PeproTech) + SCF (100 ng/ml; PeproTech) + TGFα (5ng/ml; Sigma) + TGFβ1 (10 ng/ml R & D Systems) + endothelin ( $1 \times 10^{-7}$  M; Sigma) + dexamethasone ( $5 \times 10^{-7}$ ; Sigma) + sphingosine ( $2.5 \times 10^{-7}$  M; Sigma). Alternatively, instead of sphingosine, other PKC inhibitors can be used. The cells differentiated in cells having a cardiac phenotype, as described in previous examples.

20

### Example 15

Instead of using culture conditions to promote cardiac differentiation as in Example 14, the aggregates of typical, myeloid-lymphoid stem cells were cultured under conditions that promoted a skeletal muscle phenotype. Specifically, the aggregates isolated by the methods described in Example 14 were transferred to a tissue culture dish containing Iscove's Modified Dulbecco's Medium (Gibco/BRL) + 20% fetal bovine serum (Sigma). The cells that released from the aggregates, attached to the

25



- plastic, and exhibited a fibroblast morphology were collected and plated into fibronectin-coated (Human plasma fibronectin at  $10\mu\text{g}/\text{cm}^2$ ; Collaborative) tissue culture plastic vessels containing Iscove's Modified Dulbecco's Medium (Gibco/BRL)+ 20% fetal bovine serum (Sigma) +  $50\mu\text{M}$  2-ME + retinoic acid ( $2 \times 10^{-8}$  M; Sigma) +
- 5 bFGF (100 ng/ml; PeproTech) + IL-2 +IL-15 (both at 10 ng/ml; PeproTech) + SCF (100 ng/ml; PeproTech) + IGF-1 (50 ng/ml; Sigma) + ECGS (100  $\mu\text{g}/\text{ml}$ ; Sigma) + PDGF (10 ng/ml; Sigma) + 3,3',5-triiodo-L-thyronine (20ng/ml; Sigma) + cAMP ( $5 \times 10^{-4}$  M; Sigma) + angiotensin II ( $1 \times 10^{-7}$  M; Sigma) + TGF $\beta$ 1 (10 ng/ml; R & D Systems) + endothelin ( $1 \times 10^{-7}$  M; Sigma) + dexamethasone ( $5 \times 10^{-7}$ ; Sigma) +
- 10 hyaluronic acid (100 $\mu\text{g}/\text{ml}$ ; Sigma). Cells cultured in this defined medium showed a skeletal muscle phenotype, as demonstrated by reactivity to antibodies for sarcomeric myosin heavy chain and MyoD.

#### Example 16

15

- As described in Example 11, nonadherent bone marrow stem cells are isolated from adult mice (C57BL/6) and labeled with the vital dye PKH26-GL. Labeled HSC cells are microinjected into the leg muscle of a 12-week old C57BL/6 mice. Two weeks later, the animals are sacrificed, and the injected muscle is isolated, cryopreserved, and
- 20 sectioned for immunohistochemistry. The tissue is analyzed to determine whether nonadherent HSCs incorporated into the skeletal muscle tissue, and whether the cells expressed skeletal muscle-specific markers, such as MyoD or skeletal muscle-specific myosin. Furthermore, the number of vital dye labeled cells that also show a skeletal muscle morphology (i.e., multinucleated and striated) or skeletal muscle-specific
- 25 markers are determined. These results are designed to show that adult bone marrow cells can be engrafted successfully into adult skeletal muscle tissue and that a population of the HSC cells undergo skeletal muscle cell differentiation within the permissive environment.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

5

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

10

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**What is claimed is:**

1. A method of promoting differentiation of a hematopoietic stem cell into a cell of non-hematopoietic lineage, comprising
  - (a) obtaining a hematopoietic stem cell with non-hematopoietic potential from a donor and
  - (b) contacting the stem cell with a growth factor or combination of growth factors, thereby promoting differentiation of the hematopoietic stem cell into a cell of non-hematopoietic lineage.
2. The method of claim 1, wherein the hematopoietic stem cell is a bone marrow cell.
3. The method of claim 1, wherein the hematopoietic stem cell is a blood cell.
4. The method of claim 1, wherein the hematopoietic stem cell is a myeloid-lymphoid stem.
5. The method of claim 1, wherein the donor is a nonembryonic donor.
6. The method of claim 1, wherein the cell of non-hematopoietic lineage is a cardiomyocyte.
7. The method of claim 1, wherein the cell of non-hematopoietic lineage is a skeletal muscle cell.



8. A. process of promoting the differentiation of a cardiomyocyte from a stem cell, comprising:
  - (a) obtaining a stem cell with cardiomyocyte potential from a nonembryonic donor; and
  - (b) contacting the stem cell with a growth factor or combination of growth factors, thereby promoting differentiation of the stem cell into the cardiomyocyte.
9. The process of claim 8, wherein the stem cell is non-immortalized.
10. The process of claim 8, wherein the stem cell is a bone marrow cell.
11. The process of claim 8, wherein the stem cell is a blood cell.
12. The process of claim 8, wherein the donor is a mammal.
13. The process of claim 12, wherein the mammal is a human.
14. The process of claim 8, wherein the stem cell is a hematopoietic stem cell.
15. The process of claim 14, wherein the hematopoietic stem cell is a myeloid-lymphoid stem cell.
16. The process of claim 8, wherein the step of contacting the stem cells with a growth factor or combination of growth factors is *in vitro*.

17. The process of claim 8, wherein the growth factor or combination of growth factors is selected from the group consisting of retinoic acid, stem cell factor, basic fibroblast growth factor, acidic fibroblast growth factor, endothelial cell growth factor, fibroblast growth factor-4, endothelin-1, interleukin, transforming growth factor alpha (TGF $\alpha$ ), transforming growth factor beta (TGF $\beta$ ), GM-CSF, IGF-1, platelet derived growth factor, bone morphogenic factor-4, and Wnt.
18. The process of claim 8, further comprising contacting the stem cell with a factor or combination of factors selected from the group consisting of dexamethasone, hyaluronic acid, 3,3',5-triiodo-L-thyronine, cAMP, angiotensin II, mannose,  $\beta$ -mercaptoethanol, and a Protein Kinase C (PKC) inhibitor.
19. A population of cardiomyocytes derived by the process of claim 8.
20. The population of claim 19, wherein the cardiomyocytes are noncontracting.
21. The population of claim 20, wherein the cardiomyocytes are capable of contraction upon inducement.
22. A. process of promoting the differentiation of skeletal muscle cell from a stem cell, comprising:
  - (a) obtaining a stem cell with skeletal muscle cell potential from a nonembryonic donor; and
  - (b) contacting the stem cell with a growth factor or combination of growth factors, thereby promoting differentiation of the stem cell into the skeletal muscle cell.

23. The process of claim 22, wherein the stem cell is non-immortalized.
24. The process of claim 22, wherein the stem cell is a bone marrow cell.
25. The process of claim 22, wherein the stem cell is a blood cell.
26. The process of claim 22, wherein the donor is a mammal.
27. The process of claim 26, wherein the mammal is a human.
28. The process of claim 22, wherein the stem cell is a hematopoietic stem cell.
29. The process of claim 28, wherein the hematopoietic stem cell is a myeloid-lymphoid stem cell.
30. The process of claim 22, wherein the step of contacting the stem cells with a growth factor or combination of growth factors is *in vitro*.
31. The process of claim 22, wherein the growth factor or combination of growth factors is selected from the group consisting of retinoic acid, basic fibroblast growth factor, interleukin (e.g., IL-2 and/or IL-15), stem cell factor, IGF-I, endothelial cell growth factor, platelet derived growth factor, angiotensin II, TGF $\beta$ , and endothelin.
32. The process of claim 22, further comprising contacting the stem cell with a factor or combination of factors selected from the group consisting of dexamethasone, 3,3',5-triiodo-L-thyronine, cAMP,  $\beta$ -mercaptoethanol, and hyaluronic acid.

33. A population of skeletal muscle cells derived by the process of claim 22.
34. A composition comprising:
  - (a) stem cells with cardiomyocyte potential obtained from a nonembryonic donor; and
  - (b) retinoic acid, basic fibroblast growth factor, fibroblast growth factor-4, Wnt, bone morphogenic protein-4, and either acidic fibroblast growth factor, and dexamethasone.
35. The composition of claim 34, wherein the stem cells are bone marrow cells.
36. The composition of claim 34, wherein the donor is a mammal.
37. The composition of claim 36, wherein the mammal is a human.
38. The composition of claim 34, wherein the stem cells are hematopoietic stem cells.
39. The composition of claim 38, wherein the hematopoietic stem cells are myeloid-lymphoid stem cells.
40. A composition comprising:
  - (a) stem cells with skeletal muscle potential obtained from a nonembryonic donor; and

- (b) basic fibroblast growth factor, SCF, TGF- $\beta$ , and endothelin.
- 41. The composition of claim 40, wherein the stem cells are bone marrow cells.
  - 42. The composition of claim 40, wherein the donor is a mammal.
  - 43. The composition of claim 42, wherein the mammal is a human.
  - 44. The composition of claim 40, wherein the stem cells are hematopoietic stem cells.
  - 45. The composition of claim 44, wherein the hematopoietic stem cells are myeloid-lymphoid stem cells.
  - 46. A method of determining whether stem cells of unknown cardiomyocyte potential can be promoted to differentiate into cardiomyocytes, comprising:
    - (a) contacting the stem cells with a growth factor or combination of growth factors under conditions that allow the cells to differentiate; and
    - (b) determining the presence of differentiated cells having one or more characteristics of cardiomyocytes.
  - 47. The method of claim 46, wherein the growth factors or combination of growth factors is selected from the group consisting of retinoic acid, stem cell factor, basic fibroblast growth factor, acidic fibroblast growth factor, endothelial cell growth factor, fibroblast growth factor-4, endothelin-1, interleukin, transforming

growth factor alpha (TGF $\alpha$ ), transforming growth factor beta (TGF $\beta$ ), GM-CSF, IGF-1, platelet derived growth factor, bone morphogenic factor-4, and Wnt.

48. The method of claim 46, further comprising contacting the stem cell with a factor or combination of factors selected from the group consisting of dexamethasone, hyaluronic acid, 3,3',5-triiodo-L-thyronine, cAMP, angiotensin II, mannose,  $\beta$ -mercaptoethanol, and a Protein Kinase C (PKC) inhibitor.
49. A method of determining whether stem cells of unknown skeletal muscle cell potential can be promoted to differentiate into skeletal muscle cells, comprising:
  - (a) contacting the stem cells with a growth factor or combination of growth factors under conditions that allow the cells to differentiate; and
  - (b) determining the presence of differentiated cells having one or more characteristics of skeletal muscle cells.
50. The method of claim 49, wherein the growth factors or combination of growth factors is selected from the group consisting of retinoic acid, basic fibroblast growth factor, interleukin, stem cell factor, IGF-1, endothelial cell growth factor, platelet derived growth factor, angiotensin II, TGF $\beta$ , and endothelin.
51. The method of claim 49, further comprising contacting the stem cell with a factor or combination of factors selected from the group consisting of dexamethasone, 3,3',5-triiodo-L-thyronine, cAMP,  $\beta$ -mercaptoethanol, and hyaluronic acid.

52. A method of treating a subject with heart disease or injury, comprising transplanting the cardiomyocytes of claim 19 into the cardiac muscle of the subject, thereby improving cardiac function.
53. The method of claim 52, wherein the stem cells are bone marrow cells.
54. The method of claim 52, wherein the stem cells are hematopoietic stem cells.
55. The method of claim 52, wherein the hematopoietic stem cells are myeloid-lymphoid stem cells.
56. The method of claim 52, wherein the transplanted cardiomyocytes become integrated into the cardiac muscle of the subject.
57. The method of claim 52, wherein the donor is the subject being treated.
58. The method of claim 52, wherein the subject is a mammal.
59. The method of claim 58, wherein the mammal is a human.
60. A method of treating a subject with heart disease or injury, comprising transplanting stem cells with cardiomyocyte potential into the cardiac muscle of the subject, thereby improving cardiac function.
61. The method of claim 60, wherein the stem cells are bone marrow cells.
62. The method of claim 60, wherein the stem cells are hematopoietic stem cells.

63. The method of claim 62, wherein the hematopoietic stem cells are myeloid-lymphoid stem cells.
64. The method of claim 60, wherein the transplanted stem cells with cardiomyocyte potential become integrated into the cardiac muscle of the subject.
65. The method of claim 60, wherein the donor is the subject being treated.
66. The method of claim 60, wherein the subject is a mammal.
67. The method of claim 66, wherein the mammal is a human.
68. A method of treating a subject with muscle disease or injury, comprising transplanting the skeletal muscle cells of claim 33 into the skeletal muscle of the subject, thereby improving cardiac function.
69. The method of claim 68, wherein the stem cells are bone marrow cells.
70. The method of claim 68, wherein the stem cells are hematopoietic stem cells.
71. The method of claim 70, wherein the hematopoietic stem cells are myeloid-lymphoid stem cells.
72. The method of claim 68, wherein the transplanted skeletal muscle cells become integrated into the skeletal muscle of the subject.
73. The method of claim 68, wherein the donor is the subject being treated.



74. The method of claim 68, wherein the subject is a mammal.
75. The method of claim 74, wherein the mammal is a human.
76. A method of treating a subject with skeletal muscle injury or disease, comprising transplanting stem cells with skeletal muscle potential into the skeletal muscle of the subject, thereby improving muscle function.
77. The method of claim 76, wherein the stem cells are bone marrow cells.
78. The method of claim 76, wherein the stem cells are hematopoietic stem cells.
79. The method of claim 78, wherein the hematopoietic stem cells are myeloid-lymphoid stem cells.
80. The method of claim 78, wherein the transplanted stem cells with skeletal muscle potential become integrated into the skeletal muscle of the subject.
81. The method of claim 78, wherein the donor is the subject being treated.
82. The method of claim 78, wherein the subject is a mammal.
83. The method of claim 78, wherein the mammal is a human.

## SEQUENCE LISTING

<110> MUSC FOUNDATION FOR RESEARCH DEVELOPMENT

<120> CELLS OF NON-HEMATOPOIETIC LINEAGE,  
INCLUDING CARDIOMYOCYTES AND SKELETAL MUSCLE CELLS, DERIVED  
FROM HEMATOPOIETIC STEM CELLS AND METHODS OF MAKING AND  
USING SAME

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 99/21916

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/06 C12N1/38 A61K35/34

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included, in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FERRARI G ET AL: "Muscle regeneration by bone marrow -derived myogenic progenitors 'see comments! 'published erratum appears in Science 1998 Aug 14;281(5379):923!" SCIENCE, US, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE,, vol. 279, page 1528-1530 XP002098163 ISSN: 0036-8075 the whole document --- -/--	1,2,7,8, 10,12, 14,22, 24,26, 28,33, 49, 76-78, 80,82

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"Z" document member of the same patent family

Date of the actual completion of the international search

27 January 2000

Date of mailing of the international search report

04/02/2000

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/21916

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WAKITANI S ET AL., : "Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine." MUSCLE &amp; NERVE, vol. 18, no. 12, 1995, page 1417-1426 XP000869895</p> <p>page 1418 page 1420, left-hand column, paragraph 2 -page 1421, right-hand column, paragraph 1 discussion</p> <p style="text-align: center;">---</p>	<p>1,2,7, 22,24, 26,28, 30,31, 33,49, 50, 76-78, 80-82</p>
X	<p>KLUG M G ET AL: "GENETICALLY SELECTED CARDIOMYOCYTES FROM DIFFERENTIATING EMBRYONIC STEM CELLS FORM STABLE INTRACARDIAC GRAFTS" JOURNAL OF CLINICAL INVESTIGATION,US,NEW YORK, NY, vol. 98, no. 1, 1 July 1996 (1996-07-01), page 216-224 XP000670716 ISSN: 0021-9738 cited in the application the whole document</p> <p style="text-align: center;">---</p>	<p>1,6,46, 60,64</p>
X	<p>EISENBERG C A AND MARKWALD R R: "Mixed cultures of avian blastoderm cells and the quail mesoderm cell line QCE-6 provide evidence for the pluripotentiality of early mesoderm" DEVELOPMENTAL BIOLOGY, vol. 191 (2), 15 November 1997 (1997-11-15), page 167-181 XP000866328 cited in the application the whole document</p> <p style="text-align: center;">---</p>	<p>1,6</p>
X	<p>EISENBERG C A ET AL., : "Wnt-11 is expressed in early avian mesoderm and required for the differentiation of the quail mesoderm cell line QCE-6. " DEVELOPMENT, vol. 124, no. 2, 1997, pages 525-536, XP000865667 cited in the application the whole document</p> <p style="text-align: center;">---</p>	<p>1,6</p>
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/21916

**C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT.**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MURRY C E ET AL: "Skeletal myoblast transplantation for repair of myocardial necrosis" JOURNAL OF CLINICAL INVESTIGATION, US, NEW YORK, NY, vol. 98, no. 11, 1 December 1996 (1996-12-01), page 2512-2523 XP002078686 ISSN: 0021-9738 the whole document ---	22,26, 30,31, 33,40, 68,72,74
A	LEOR J ET AL: "Transplantation of fetal myocardial tissue into the infarcted myocardium of rat. A potential method for repair of infarcted myocardium?" CIRCULATION, US, AMERICAN HEART ASSOCIATION, DALLAS, TX, vol. 94, no. 9, SUPPL. 1 November 1996 (1996-11-01), page 11332-11336 XP002078687 ISSN: 0009-7322 the whole document ---	22,26, 30,31, 33,40, 42,68, 72,74
A	BRUDER S P ET AL: "MESENCHYMAL STEM CELLS IN BONE DEVELOPMENT, BONE REPAIR, AND SKELETAL REGENERATION THERAPY" JOURNAL OF CELLULAR BIOCHEMISTRY, US, NEW YORK, NY, vol. 56, 1994, page 283-294 XP002913340 page 283-284; figure 1 ---	1,2
P,X	KESSLER PD; BYRNE BJ: "Myoblast cell grafting into heart muscle: cellular biology and potential applications" ANNU REV PHYSIOL (UNITED STATES), vol. 61, 1999, page 219-42 XP000865708 the whole document ---	1-83
P,X	FERRARI G ET AL.: "Bone marrow transplantation as source of myogenic progenitors: Differentiation of stem cells into muscle." CELL TRANSPLANTATION, vol. 8, no. 2, April 1999 (1999-04), page p195 XP000869901 abstract --- -/-	1,2,7, 22,24, 28,33, 49, 76-78, 80,82

# INTERNATIONAL SEARCH REPORT

Int. l. Application No  
PCT/US 99/21916

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>TOMITA S ET AL., : "Autotransplanted mesenchymal stem cells improve function after a myocardial infarction" CIRCULATION , vol. 98, no. (17 SUPPL.), 27 October 1998 (1998-10-27), page pI200 XP000870314</p> <p>abstract</p>	<p>1,2,5,6, 8,10,12, 14,16, 19,21, 52-54, 56,58, 60-62, 64,66</p>
P,X	<p>WO 99 03973 A (OSIRIS THERAPEUTICS INC ;GORDON STEPHEN L (US); MACKAY ALASTAIR MO) 28 January 1999 (1999-01-28)</p> <p>abstract page 3, line 5-25 page 4, line 10-20: example 1</p>	<p>1,6,8, 12,13, 19,34, 46,47, 52,60</p>
T	<p>TOMITA S ET AL., : "Autologous transplantation of bone marrow cells improves damaged heart function." CIRCULATION , vol. 100 (19 SUPPL.), 9 November 1999 (1999-11-09), page pII247-II256 XP000869909</p> <p>the whole document</p>	<p>1,2,6,8, 10,12, 14,16, 17,19, 21,46, 52-54, 56,58, 60,61, 64,66</p>

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/21916

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 52-83  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 52-83 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/21916

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9903973 A	28-01-1999	AU 8401498 A	10-02-1999